Universidade de Aveiro Departamento de Biologia
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Guilherme Marques Teixeira

Benefits of a marine macroalgae enriched-diet on the biochemical status of the eyes and brain in fish exposed to formalin.

Benefícios de uma dieta rica em macroalgas marinhas para a condição bioquímica de olhos e cérebro de peixes expostos a formalina.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Doutora Patrícia Alexandra Oliveira Pereira Kowalski, investigadora em Pós-doutoramento da Universidade de Aveiro, e coorientado pelo Doutor Mário Guilherme Garcês Pacheco, Professor auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro

“Deus quer, o homem sonha, a obra nasce.”

Fernando Pessoa

o júri

Presidente

Professor doutor Ulisses Manuel de Miranda Azeiteiro
Professor associado com agregação, Universidade de Aveiro

Doutora Vera Lúcia de Almeida Maria
Bolseira de Pós-Doutoramento, Universidade de Aveiro

Doutora Patrícia Alexandra Oliveira Pereira Kowalski
Bolseira de Pós-Doutoramento, Universidade de Aveiro

agradecimentos

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palavras-chave

Alimentos funcionais; Aquacultura; Estruturas neurosensoriais; Formalina; Macroalgas; *Sparus aurata*

resumo

Vários benefícios para a saúde humana têm vindo a ser associados a compostos bioactivos de macroalgas marinhas (MM), compreendendo um aumento da proteção contra o *stress* oxidativo e perda de sinapses, que são processos tipicamente envolvidos em distúrbios neurodegenerativos. De facto, os benefícios das macroalgas têm sido amplamente investigados com enfoque nas condições neurológicas (entre outras) em humanos, enquanto que as suas vantagens na saúde de peixes em aquacultura permanecem pouco exploradas. Tendo em conta as semelhanças das vias neurológicas entre peixes e mamíferos, assim como as analogias estruturais do cérebro e dos órgãos sensoriais, são expectáveis efeitos benéficos de uma dieta enriquecida em MM também em peixes. Estes benefícios são, provavelmente, mais evidentes quando os peixes estão sujeitos a condições exógenas que possam desafiar o seu estado neurológico, como poderá ser o caso da formalina (desinfectante usado frequentemente em aquacultura), que já foi associada a efeitos neurotóxicos em mamíferos. A presente dissertação foi desenhada neste contexto, com o objectivo de abordar, e pela primeira vez, a proteção antioxidante e da neurotransmissão conferida por uma dieta suplementada com MM em olhos e cérebro de dourada (*Sparus aurata*) em condições de base, assim como após exposição a formalina. Com este objectivo, os peixes foram alimentados durante 2 meses com uma dieta suplementada com MM [incorporação total de 5 %, com as espécies *Fucus vesiculosus*, *Gracilaria gracilis* e *Ulva rígida* em partes iguais – grupo de peixes suplementado com algas (A)], enquanto que os peixes não suplementados foram alimentados com uma ração padrão/*standard* (S) (alimentação sem MM). De seguida, os dois grupos com diferentes dietas de base foram sujeitos a um banho de formalina (F) durante 1 hora (grupos SF e AF). O tratamento com formalina foi repetido 2 dias mais tarde. Os grupos controlo, não expostos a formalina, foram mantidos ao longo da experiência (S e A), que teve uma duração de 18 dias após a 1ª exposição. Os peixes dos diferentes grupos (S, A, SF, AF) foram sacrificados ao fim de 4 e 18 dias após a exposição à formalina, sendo que os olhos e o cérebro foram recolhidos para a determinação dos seguintes parâmetros bioquímicos: (i) antioxidantes enzimáticos (catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa - s - transferase (GST), glutationa redutase (GR) e não enzimáticos (glutationa total (GSHt)); (ii) indicadores de dano (peroxidação lipídica – LPO e oxidação proteica (PO); (iii) actividade da acetilcolinesterase (AChE), usada como um indicador da neurotransmissão. Além disso, foi feita uma avaliação simplificada da condição geral dos peixes. Não foram registadas alterações significativas no peso dos peixes, comprimento total e índice de condição ao longo do período experimental, para qualquer dos tratamentos. Este resultado sugere que as MM podem ser incluídas na dieta da dourada sem comprometer a sua taxa de crescimento e, portanto, sem vir a ter um efeito negativo nos dividendos associados à produção de peixe. Em contrapartida, foram registadas algumas alterações significativas nos parâmetros bioquímicos medidos nos olhos e cérebro dos peixes que estiveram 2 meses sob uma dieta enriquecida em MM, embora essas alterações tenham sido difíceis de interpretar por associação direta ao tipo de dieta fornecida. Por outro lado, aos 4 dias observou-se um aumento da peroxidação lipídica e da oxidação proteica nos olhos dos peixes suplementados com MM e não expostos à formalina (grupo controlo – A). Este resultado levantou algumas dúvidas sobre os benefícios de uma dieta enriquecida em MM *per se*, ou seja em ausência de um desafio pró-oxidante. De um modo geral, aos 4 e 18 dias após a exposição à formalina, foram registados padrões de variação idênticos para os antioxidantes, peroxidação lipídica e a atividade da AChE no cérebro de peixes suplementados com MM, representados por aumentos significativos nas condições A e AF. Esta semelhança de padrões de variação parece indicar que uma dieta rica em MM poderá modular os mecanismos de defesa no cérebro de *S. aurata*. Foram, contudo, registadas algumas exceções 18 dias após a exposição a formalina. É de salientar que os efeitos da suplementação com MM no estado pró-oxidante dos olhos e cérebro, assim como na neurotransmissão, se tornaram mais evidentes após a exposição dos peixes a formalina. Designadamente, 4 dias após a exposição a formalina, os peixes alimentados com uma dieta suplementada em MM (AF) apresentaram uma melhoria na defesa antioxidante não enzimática nos olhos, tal como demostrado pelo aumento dos níveis da GSHt.

Por conseguinte, uma dieta enriquecida em MM preveniu a ocorrência de oxidação das proteínas, assim como o aumento de AChE que terá sido promovido pela formalina nos olhos de peixes sob uma dieta *standard* (SF). Dezoito dias mais tarde, essa proteção terá continuado a manifestar-se nos olhos dos peixes alimentados com MM, tal como foi traduzido pela sua capacidade em prevenir a depleção de GSHt induzida pela formalina, assim como a ocorrência de *stress* oxidativo (aumento de PO e LPO) e aumento de sinais de neurotoxicidade (pela inibição de AChE), tal como foi registado nos olhos dos peixes sob uma dieta *standard* (grupo SF). Os resultados apontam para um impacto da formalina no cérebro dos peixes mais tardio do que aquele que foi registado nos olhos, dado que foram observados efeitos unicamente 18 dias após a exposição. Neste período, a peroxidação lipídica foi prevenida no cérebro dos peixes suplementados com MM (AF), o que poderá estar associado ao aumento notório de GSHt (que também foi registado em peixes alimentados com dieta padrão – SF). A formalina induziu um efeito tardio (18 dias) na AChE, tal como demostrado pelo aumento da sua actividade. Este resultado sugere um desequilíbrio na homeostase colinérgica do cérebro, que não terá sido prevenido pelo enriquecimento em MM. De um modo geral, os resultados da actividade de AChE no cérebro não apontaram para benefícios das MM na neurotransmissão. Em conclusão, a formalina teve um efeito superior nos olhos de *S. aurata* do que no cérebro, tendo sido esse efeito registado mais precocemente. A água é a via de exposição dos peixes à formalina, o que poderá contribuir para explicar o resultado anterior. As alterações fisiológicas induzidas pela formalina colocaram em evidência as propriedades protetoras que uma dieta enriquecida em MM poderá ter na função neuro-sensorial de peixes. Contudo, é necessária ainda mais investigação, em particular no contexto da aquacultura, relativamente às propriedades das MM, especificamente ao nível do cérebro e estruturas sensoriais após exposição dos peixes a formalina.

keywords

Macroalgae; Functional foods; Fish farming; Formalin; Neurosensory structures; *Sparus aurata*

abstract

Many health benefits have been associated with bioactive compounds of marine macroalgae (MM), including protection against oxidative stress and synaptic loss that are hallmarks of human neurodegenerative disorders. While the benefits of macroalgae have been largely explored with a focus on human health associated with neurological status (among others), advantages to farmed fish health remain elusive. Based on similarities of neurological pathways between fish and mammals, as well as on identical structures of the brain and sensory organs, beneficial effects of MM-enriched feeds can be expected on fish. These benefits are probably more evident when fish are under exogenous challenging conditions to their neurological status, as can be the case of formalin exposure (a frequently used disinfectant in aquaculture), which has been associated with neurotoxic effects in mammals. The current dissertation was designed under this context, aiming to address, for the first time, the antioxidant and neurotransmission protection afforded by a MM-enriched diet to the eyes and brain of the gilthead seabream (*Sparus aurata*) under baseline conditions, as well as after formalin exposure. For this purpose, fish were fed for 2 months with a MM-enriched feed [total incorporation of 5%, with the species *Fucus vesiculosus*, *Gracilaria gracilis* and *Ulva rigida* in equal parts - algae supplementation fish group (A)], while non-supplemented fish were fed with a standard diet (S) (without MM). Then, both dietary background groups were subjected to a formalin (F) bath for 1 hour (fish groups AF and SF). Such formalin treatment was repeated 2 days later. Control groups, unexposed to formalin, were maintained along the experiment (A and S) that lasted 18 days after the first formalin exposure. During the whole experiment, fish were fed twice a day, while water quality was monitored daily. Fish of the different groups (A, S, AF, SF) were sacrificed 4 and 18 days after the formalin exposure, with the eyes and brain being collected for the determination of the following biochemical parameters: (i) enzymatic (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione - s - transferase (GST), glutathione reductase (GR) and non-enzymatic antioxidants (total glutathione (GSHt)); (ii) damage indicators (lipid peroxidation – LPO; protein oxidation - PO); (iii) acetylcholinesterase (AChE) activity used in this research as a proxy of neurotransmission. Besides that, a gross assessment of fish condition was made. No significant changes were recorded on weight, total length and condition factor of the fish over the experimental time, regardless the treatment. This suggests that MM can be included in gilthead seabream diet without compromising growth rates, and therefore with no detrimental effect on fish production revenues. Differently, two months after macroalgae supplementation, there were a few significant alterations on the eyes and brain biochemical parameters, although difficult to be straightforward associated to the different dietary backgrounds afforded to *S. aurata*. Moreover, lipid peroxidation and protein oxidation in the eyes of fish supplemented with MM and non-exposed to formalin (control group - A) was recorded at day 4, fetching some doubts on the benefits of the macroalgae-enriched diet *per se*, *i.e*., in the absence of a pro-oxidant challenge. Four and 18 days after the formalin exposure, the antioxidants, lipid peroxidation and the AChE activity showed similar variations in the brain of the supplemented fish, represented by significant increases in A and AF conditions. This pattern similarity may indicate that MM dietary supplementation can be important to modulate the brain defence mechanisms in *S. aurata*. Yet, in the 18 days period it was reported a few exceptions. However, upon formalin exposure, the effects of MM supplementation on the pro-oxidant status and neurotransmission of those organs were remarkable. Four days after formalin exposure, fish fed with a macroalgae-supplemented diet (AF) displayed an improvement on the non-enzymatic antioxidant defense of the eyes against formalin, as depicted on the increase of total glutathione levels (GSHt). Accordingly, MM-enriched diet impaired the occurrence of protein oxidation and AChE enhancement promoted by formalin in the eyes of fish fed with a standard diet (SF). After 18 days, it was evident a persistence of the macroalgae protection in the eyes, as depicted on the capacity to avoid formalin-induced GSHt depletion, oxidative stress (as PO and LPO increases) and neurotoxicity (as AChE inhibition) observed in the eyes of non-supplemented fish (SF). A delayed impact of formalin was perceived in the brain in comparison to the eyes, since formalin effects were detected only 18 days after formalin exposure.

By this time, lipid peroxidation was prevented in fish supplemented with macroalgae (AF), which cannot be dissociated from a notorious increase on GSHt content (that also occurred in fish fed with standard diet - SF). Formalin induced a late effect (day 18) on AChE as displayed by its activity increase, suggesting an imbalance on the cholinergic homeostasis, which was not prevented by the macroalgae enrichment. Results on AChE in the brain did not unveil the benefits of MM on neurotransmission. In conclusion, formalin presents a higher and earlier effect on *S. aurata* eyes when compared with the brain tissue, probably associated with its exposure route (water). The physiological alterations provoked by the formalin brought into light the shielding proprieties of MM supplementation in the fish neurosensory function. However, the MM beneficial proprieties deserve more research under the aquaculture context, specifically at the level of neuronal and sensory effects of formalin.

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1. INTRODUCTION

1.1. Marine macroalgae as a source of potential health-promoting compounds

Marine macroalgae (MM) can be subdivided in 3 different groups based on their pigmentation, *i.e*, brown (Phaeophyta), red (Rhodophyta), or green (Chlorophyta) (Figure 1). The pigment responsible for the brown color of the Phaeophyta species is fucoxanthin, the red color of the Rhodophyta species comes from phycobilins, whiles there are several pigments responsible for the green color (*e.g*., chlorophyll a and b, carotenes and xanthophylls) in the Chlorophyta species (Øverland et al., 2018). MM have been largely investigated both for novel ingredients and as a health-promoting food (Patarra et al., 2011; Mohamed et al., 2012). Interestingly, archaeological studies underpinned that in southern Chile, MM have been consumed since the 14,000 years, while in the 50s of the 20th century the MM production in aquaculture had a breakthrough in Asia (Kim et al., 2017). In Europe, MM aquaculture is under development since 90's of the last century with a current production of 54,000 tons per year, almost exclusively directed to human consumption, whereas just a small portion is used in fish diet (FAO, 2017). Worldwide, the MM aquaculture production represents 20 % of the total marine aquaculture production by weight (Bjerregaard et al., 2016).

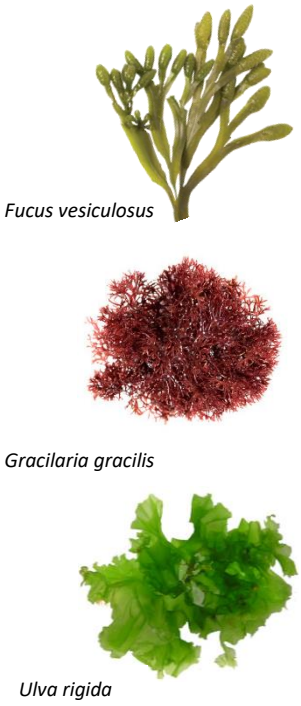

Figure 1: Marine macroalgae species, potentially used in a macroalgae enriched-diet or as a source of functional compounds. (http://www.algaebase.org)

Marine macroalgae can be considered a functional food, meaning that they have ingredients that confer an additional function to the food (usually related to health-promotion or disease prevention) (Kaur and Das, 2011; Wells et al., 2016). Indeed, MM have a number of characteristics that make them valuable as a functional food. They have low cytotoxicity, anti-inflammatory and antioxidant capacities, and are able to hinder cellular death, while having a low production cost (*e.g*. Pangestuti and Kim, 2011). Besides supporting the exploitation of MM by the food industry (about 80 % of the world production), those properties are esteemed to the pharmaceutical and cosmetic production, where currently MM have also been widely used (Pangestuti and Kim, 2011).

In general, MM are rich in soluble dietary fibres, proteins, antioxidants, vitamins and polyunsaturated fatty acids (PUFAs), while having a low caloric value (Rupérez and Saura-Calixto, 2001). Marine macroalgae have a wide range of antioxidant compounds, but there is still a poor knowledge on the hypothetical extension of their benefits to organisms upon consumption, namely in humans (Wells et al., 2016). Hypothetically, MM antioxidants could act by limiting reactive oxygen species (ROS) in the digestive tract, thus decreasing oxidative stress on the gut microbiome and epithelial cells. Additionally, MM antioxidants could be transported into the blood for distribution throughout the body. In humans, the evidence of a direct transport is very limited, since there are no systemic studies on the digestive uptake of these compounds. In fish, it was showed that MM bioactive compounds affect the gut morphology and thickness of the goblet cells inducing changes in the digestion and absorption of nutrients in Nile tilapia (Silva et al., 2015) and rainbow trout (Heidarieh et al., 2012). However, there are no evidences in the way of how and if the enzymatic antioxidants are absorbed. Lipids are essential for all living organisms as components of membranes, energy storage compounds, and as cell signaling molecules (Eyster, 2007; Muro et al., 2014). MM could be a source of lipids to other organisms, namely of the long-chain polyunsaturated fatty acids and carotenoids. The most important PUFAs are the essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as, their precursors α-linolenic acid (ALA) and docosapentaenoic acid (DHA) (Cottin et al., 2011). However, humans and other animals cannot convert ALA to EPA and DHA at required level. Thus, they need a food source rich in these essential fatty acids (Wells et al., 2016). Furthermore, there are evidences that enhanced DHA intake may improve the child cognitive performance, as well as, the visual acuity (Jensen et al. 2005, 2010; Imhoff-Kunsch et al. 2011). Also, DHA present in the MM can also produce cardiovascular protection in humans, thought the alteration of the plasma lipoproteins (2 g algal DHA day−1 over 4.5 month) (Neff et al., 2011). The carotenoids present in the MM are capable of free radical scavenging activity *in vitro* and *in vivo* (Wells et al., 2016).

There are a wide number of beneficial effects described for MM, namely a reduction on the human blood pressure (Wada et al., 2011) and an improvement of the immune system (Zhang et al., 2017). Specifically, the fucoidans of a brown MM *Laminaria saccharina*, *Fucus vesiculosus* and *Fucus spiralis* demonstrated the capacity to induce apoptosis in cancer cell lines and to promote macrophage-induced tumor cell death (Cumashi et al., 2007). A sulphate polysaccharide of a red MM *Gigartina skottsbergii* revealed antiviral properties (Ahmadi et al., 2015), while phenols of a green MM *Ulva lactuca* have the capacity of scavenging the ROS and to confer protection against synaptic loss

(Mohamed et al., 2012). Indeed, the neuroprotective properties of MM have been also demonstrated, mostly related with their antioxidant properties, anti-neuroinflammation, together with a modulation of the cholinesterase activity (Pangestuti and Kim, 2011). Table 1 describes a number of compounds identified in MM with the previous properties in mammal models (*e.g*. rodents; mammalian cell lines). For example, fucoxanthin of *Laminaria japonica* afforded protection to rabbits' retina against the oxidative damage inflicted by the visible light (Liu et al., 2016), while positive interferences with the cholinergic, dopaminergic and serotonergic systems were also described in humans and rodents (Rico et al., 2011). Moreover, a study with rats demonstrated that fucoidan administration can act as an anti-adhesion of leukocyte-endothelial attenuating the hypoxia-ischemia that will lead to brain damage (Uhm et al., 2003).

Overall, various MM species have valuable bioactive compounds such as vitamins, minerals, proteins, polysaccharides, steroids, dietary fibers, carotenoids, saturated and polyunsaturated fatty acids (Peixoto et al., 2016) that may have several phytochemicals with potential advantages for neurodegenerative conditions (Table 1).

Table 1: Compounds of marine macroalgae with neuroprotective related properties

Compound	Macroalgae Class	Model	Reference
Dieckol	Phaeophyta	*In vitro*	Kannan et al. 2013
Docosahexaenoic acid	Chlorophyta Phaeophyta Rhodophyta	*In vitro*	Andrade et al. 2013
Eckol	Phaeophyta	Human	Yoon et al. 2008
Eckstolonol	Phaeophyta	Human	Yoon et al. 2008
Eicosapentaenoic acid	Chlorophyta Phaeophyta Rhodophyta	Rat	Lynch et al. 2007
Fucoidan	Phaeophyta	Rat	Uhm et al., 2003
Fucosterol	Phaeophyta	Human	Yoon et al. 2008
Fucoxanthin	Phaeophyta	Rat	Zhang et al. 2017
Glycerol	Chlorophyta	*In vitro*	Fang et al. 2010
Phlorofucofuroeckol	Phaeophyta	Rat	Ahn et al. 2012
Phlorofucofuroeckol A	Phaeophyta	Human	Yoon et al. 2008
Phloroglucinol	Chlorophyta	*In vitro*	Pangestuti and Kim 2011
Uridine	Chlorophyta	*In vitro*	Fang et al. 2010
2-Phloroeckol	Phaeophyta	Human	Yoon et al. 2008
7-Phloroeckol	Phaeophyta	Human	Yoon et al. 2008
6,6'-Bieckol	Phaeophyta	Human	Yoon et al. 2009

1.2. Fish sensory organs and brain: considerations on biochemical condition and physiology

Most fish have developed highly sensory organs/structures, comprising different types of receptors/sensors, namely: (i) chemoreceptors (responsible, for example, for gustation and olfaction);

(ii) photoreceptors (visual information); (iii) nociceptors (detection of noxious tissue-damaging stimuli); (iv) mechanoreceptors (mechanosensory lateral line system); (iv) electroreceptors (electrosensory lateral line system). The mechanosensory lateral line together with the auditory sense, compose the mechanical sense system that is involved in the control of fish body position.

The fish eyes are an important and highly specialize sensory organ that converts light/images into neural signals. This conversion is possible due to the existence of photoreceptors that are located in the retina (Ali et al., 1978). The eyes are located symmetrically or in the same side of the fish head and present a direct contact with the surrounding environment (Randall et al., 1997). This organ is mainly divided into: (i) cornea (outermost transparent layer of the eye); (ii) iris (controls the amount of light entering the eyes); (iii) lens (focuses light on the retina); (iv) sclera (forms the outer layer of the eyes and protects the inner structures of the eyes); (v) choroid (highly vascularized region between the sclera and the retina); (vi) retina (transparent laminar structure located at the back of the eyes) (Figure 2). The retina structure presents a colorful pigment that is called carotenoid, with the capacity to provide protection against several stressors, including UV radiation or ROS (de Carvalho and Caramujo, 2017). Carotenoids are only synthesized by macroalgae, plants, fungi and bacteria, while other organisms only obtain the necessary pool of these compounds through the diet (de Carvalho and Caramujo, 2017) (Table 1). The dietary supplementation with polyunsaturated fatty acids showed relevant benefits for the health of the eyes, in order to prevent diseases, like glaucoma and macular degeneration (Saccà et al., 2018). Furthermore, eyes are a tissue that can accumulate lipids, but the percentage of accumulated lipids, such as DHA, may vary between different fish species (Stoknes et al., 2004). In the same line, Hong et al. (2014) showed that freshwater fish can accumulate more lipids than the muscle tissue. Vitamins are a complex organic compounds present in the fish eyes structure and are essential to the normal metabolism and the lack of these compounds can lead to diseases (McDowell and Cunha, 1989). The vitamin C or ascorbic acid presents an effective antioxidant capacity (Bendich et al., 1986), which has been demonstrated in several experiments *in vitro* studies with several species, including fish. For example, vitamin C can reduce the formation of ROS, resulting in the reduction of the lipid peroxidation in *in vitro* cells (Padayatty et al., 2003).

Fish brain is relatively small in comparison with other vertebrates (generally one-fifteenth the brain mass of a similarly sized mammal) (Bone and Moore, 2009). The fish brain has main divisions that extend rostrocaudally, as following (examples of minor divisions/structures are indicated in brackets together with their function) (Evans and Claiborne, 1997) (Figure 2): (i) telencephalon (*e.g*. bulbus

olfactorius; mostly involved in olfaction) and diencephalon (*e.g*. epithalamus, thalamus and hypothalamus; mainly involved in the correlation of afferent and efferent impulses and modulation of the endocrine system); (ii) mesencephalon or midbrain (*e.g*. optic tectum and tegmentum; mainly involved in the vision and learning); (iii) metencephalon (cerebellum; mostly implicated in the coordination of muscular activities during swimming) and myelencephalon (medulla oblongata; mainly involved in sensory functions such as gustation and audition). The medulla oblongata and the tegmentum are collectively referred as brainstem (Evans and Claiborne, 1997). The spinal cord extends along the fish body. The cerebellum (motor learning and coordination, and, probably, cognition), optic tectum (orientation tasks, such as object identification and location) and telencephalon (olfaction) are examples of integrative centers in fish brain (Evans and Claiborne, 1997).

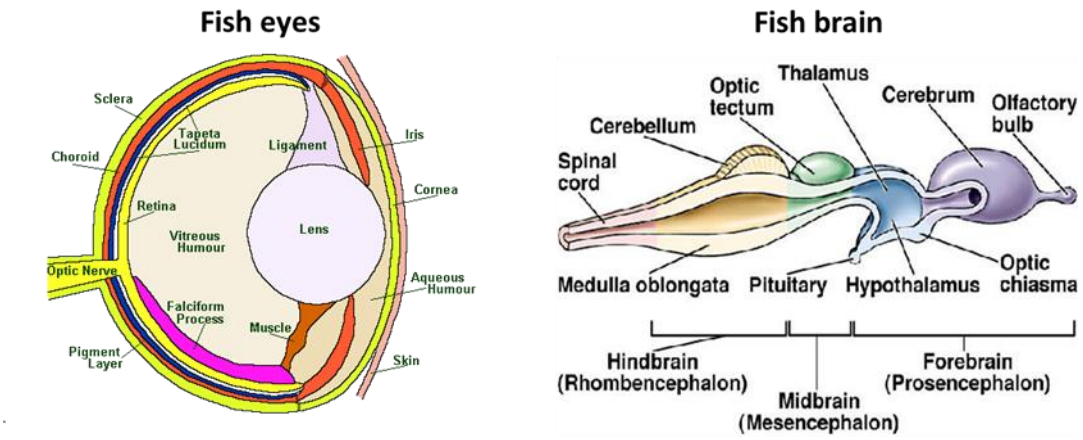

Figure 2: Schematic images of fish eyes (https://bit.ly/2Am0BFh) and brain (with the identification of the main areas distributed rostrocaudally) (https://bit.ly/2Ri985Y).

Fish brain is composed by essential fatty acids that are important for the function maintenance and may represent two-thirds of the brain weight (Singh, 2005). Docosahexaenoic acid (DHA) is an essential fatty acid distributed in the cerebral cortex (Singh, 2005). This fatty acid is important for the growth and functional development of the brain and the DHA deficiency can be associated with deficits in learning (Horrocks and Yeo, 1999). Eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) are other essential fatty acids that are important for the brain health, although less abundant than DHA (Dyall, 2015). In general, these three fatty acids (DHA, EPA and DPA) present important functions

against the cognitive decline or depressive symptoms, having a vital role in the neuroprotective capacity (Dyall, 2015).

Neurotransmission is the basis of neuronal communication, comprising a set of biochemical processes that are vulnerable to environmental toxicants exposure. The major neurotransmitter systems identified in fish were thoroughly reviewed by (Horzmann and Freeman, 2016) and are associated to the following classical transmitter substances, namely: (i) glutamate (the primary excitatory neurotransmitter and the most common in the bony fish brain); (ii) gamma aminobutyric acid (GABA - the major inhibitory neurotransmitter in the CNS); (iii) catecholamine neurotransmitters [dopamine (DA), norepinephrine (NE) and epinephrine - modulatory neurotransmitters]; (iv) serotonin (5-HT – a modulatory neurotransmitter); (v) acetylcholine (ACh - the major neurotransmitter in the parasympathetic nervous system); (vi) histamine (a non-synaptic neuromodulator); (vii) glycine (an inhibitory neurotransmitter).

In detail, acetylcholine (ACh) is a fast-acting neurotransmitter at the neuromuscular junction and in the autonomic ganglia (Figure 3). Furthermore, there is an anatomical mismatch between the sites of ACh release and the location of cholinergic receptors (Picciotto et al., 2012). This neurotransmitter has the capacity to modulate the neurological function in the brain (Picciotto et al., 2012). Acetylcholinesterase is an important part of the cholinergic nervous system in fish (Whitehead et al., 2005). Furthermore, there is a necessity for maintenance of acetylcholine levels and, acetylcholinesterase (AChE) (Figure 3) is the enzyme responsible for that process, through the break of acetylcholine into acetate and choline (Soreq, 2001; Wilkinson et al., 2004). This enzyme is a target for several toxins, contaminants or pharmaceutical that may inhibit or reactivate its function, although, the main effect is the inhibition of the AChE (Araújo et al., 2016).

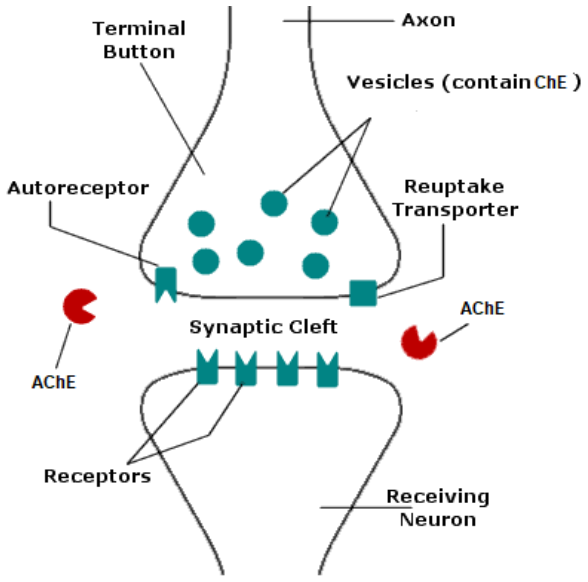

Figure 3: Basic functioning of cholinergic neurotransmission (adapted from Randall et al. (1997)).

The knowledge of the differences and similarities in the functional organization of nervous system between fish and other vertebrate groups (mammals, including) remains elusive (Evans and Claiborne, 1997). Several research challenges remain unsolved, namely regarding the homology of particular nuclei, neuronal connections, neurotransmitter distribution, as well as sensory pathways. Despite that, remarkable progresses have been made due to the investigation on the zebrafish neurobiology (Parng et al. 2002; Gerlai 2011; Rico et al. 2011; Kalueff et al. 2014; Alshabani et al. 2016). This model species shares the main neurotransmitter pathways with mammals and has similar neuroanatomy in many areas (*e.g*. spinal cord, hindbrain and retina), while some of the classical regions of the mammalian brain are not present (*e.g*. hippocampus, amygdala, and substantia nigra) with that organization (Horzmann and Freeman, 2016).

1.3. Oxidative stress and neurodegeneration in fish

Oxygen is vital for all living cells, while it is potentially dangerous in excess. Therefore, oxygen is kept under a tight check by a complex system that regulates and monitors the usage and uptake of this element. Reactive oxygen species are produced in cells, primarily as a result of the aerobic metabolism. Numerous studies have been showing the advantageous biological effects of some ROS, such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and peroxyl radical ($ROO^{\bullet}$). Important physiological functions involving ROS include the following: 1) regulation of vascular tone; 2) sensing of oxygen tension and regulation of functions that are controlled by oxygen concentration; 3) enhancement of signal transduction from various membrane receptors; 4) oxidative stress responses that ensure the maintenance of redox homeostasis (Dröge 2003). While $O_2^{\bullet-}$ is formed through one-electron reduction of O_2, H_2O_2 can be produced by the dismutation of $O_2^{\bullet-}$ (catalysed by superoxide dismutases) via the hydroperoxyl radical ($HO_2^{\bullet-}$) (Figure 4). $^{\bullet}OH$ is probably the most reactive and toxic form, which is produced by the metal ion catalysed decomposition (*e.g*. iron or copper) of H_2O_2. In mitochondria, oxygen takes part in glucose breakdown through oxidative phosphorylation, generating energy (in the form of ATP). Over 90% of cellular oxygen is consumed in mitochondria of unstressed cells, and therefore it is considered a major site of aerobic cellular ROS production (Han et al., 2001). Moreover, ROS generation occurs also by microsomal systems of the endoplasmatic reticulum (Winston et al. 1996).

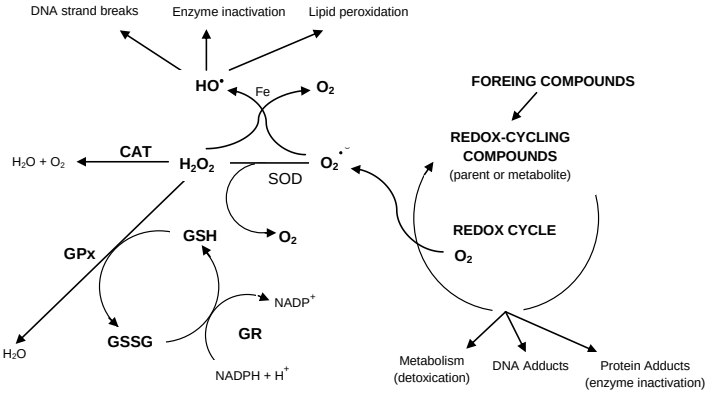

Figure 4: Antioxidant defenses dynamic in relation to ROS production under a scenario of exposure to exposure to a foreign compound to the cell with potential toxic action. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidised glutathione (adapted from Stegeman et al., 1992).

A regulated production of free radicals and the maintenance of ‘‘redox homeostasis’’ are essential for the physiological health of organisms (Ames et al., 1993). Despite that, a small proportion (2–3%) of free radicals may escape from the protective shield of antioxidant mechanisms, causing oxidative damage to biomolecules (DNA, proteins and lipids) (Halliwell and Gutteridge, 1999). The imbalance between generation and neutralization of ROS by antioxidant mechanisms is called oxidative stress (Davies, 1995) (Figure 5). During evolution, biological systems have been developing adequate enzymatic and non-enzymatic antioxidant mechanisms to protect their cellular components from oxidative damage. These include antioxidant enzymes, such as: i) superoxide dismutase (SOD); ii) catalase (CAT); iii) glutathione peroxidase (GPx); iv) glutathione reductase (GR); v) glutathione-S-transferase (GST) (Figure 4). Other molecules with antioxidant action such as glutathione, uric acid and ascorbate play also an important role in counteracting ROS (Martínez-Álvarez et al., 2005).

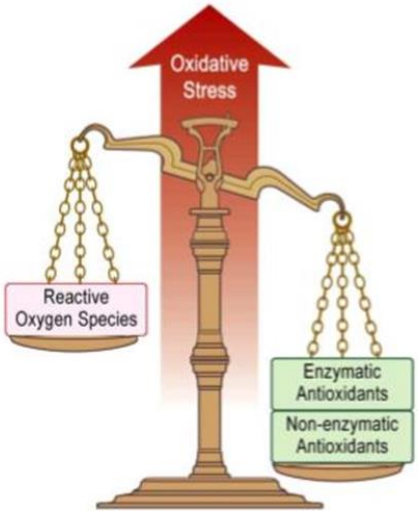

Figure 5: Oxidative stress occurs when the balance highly reactive radicals (oxidants) and antioxidants tips towards the oxidants (Adapted from Lee et al., 2010).

In detail, CAT is a heme-containing enzyme that facilitates the removal of H_2O_2, which is decomposed to O_2 and water (Figure 4) (Schrader and Fahimi, 2006). CAT employs one molecule of H_2O_2 as donor in the reduction of another H_2O_2, while peroxidases use other reductants. In animal cells, the principal peroxidase is a selenium-dependent tetrameric cytosolic enzyme (GPx) that employs reduced glutathione (GSH) as a cofactor. GPx catalyses the metabolism of H_2O_2 to water with the concomitant conversion of GSH to its oxidized form (glutathione disulfide - GSSG) (

Figure 4) (Halliwell and Gutteridge, 1999). GSTs may play a dual protective role associated to their activity on conjugation of electrophilic compounds (or phase I metabolites) with GSH (Oost et al., 2003) and can also employ GSH in the reduction of a broad range of organic hydroperoxides, but it cannot reduce H_2O_2 (Wang and Ballatori, 1998). GR catalyses the transformation of GSSG to GSH with the concomitant oxidation of NADPH to $NADP^+$ (

Figure 4). Therefore, GR maintains the GSH/GSSG homeostasis under oxidative stress conditions (Winston and Giulio 1991). Glutathione represents the bulk of the non-protein thiols of the cells. Specifically, GSH (a tripeptide of glutamine acid, cysteine and glycine) may have a dual role in detoxification, namely: (i) as a key conjugate of electrophilic intermediates (mainly via GST activity in phase II metabolism); (ii) as an important antioxidant (Stegeman et al., 1992). Exposure of aquatic organisms to environmental toxic compounds may lead either to an increase or decrease of those enzyme activities, as well as of GSH. Specifically, in fish eyes and brain it have been described significative alterations in the antioxidant system of *Liza aurata* eyes when exposed to mercury (Pereira et al. 2016). In the same species, it has already been shown variations in the brain antioxidants when the fish was exposed to mercury (Cardoso et al., 2017). Furthermore, it was recorded an alteration in GSH content in *Liza aurata* subject to an accumulation of mercury (Mieiro et al., 2011). Furthermore, a glyphosate-base herbicide roundup transorb (RDT) can alter the antioxidant defenses in the neotropical fish *Prochilodus lineatus* (Modesto and Martinez, 2010). Besides that, the antioxidant system of juvenile common carps can be affected by the hexachlorobenzene (HCB) (Song et al., 2006). Overall, these findings pointed out that these antioxidants are able to underpin a challenging condition to fish.

The biochemical and physiological effects provoked by xenobiotics in the cells have been associated with increased fluxes of oxyradicals, which may reflect the emergence of lipid and protein damage (Oost et al., 2003). The process of lipid peroxidation comprises a set of chain reactions which can influence the PUFA, given that due to the double bounds, they are very sensitive to reactions by ROS. Lipid peroxidation products may form DNA adducts giving rise to mutations and altered patterns of gene expression (Marnett, 1999). Peroxidized membranes become rigid, with the consequence of loosing permeability and integrity. Furthermore, the proteins are one of the major targets ROS (Davies, 2016). The ROS leading to protein oxidation include radical species such as hydrogen peroxide, hypochlorous acid (HOCl), ozone (O^3) and peroxynitrite ($ONOO^-$) (Ahmad, 2017). Carbonyl groups are produced on protein side chains when the proteins are oxidized (Ahmad, 2017). However, can be also introduced into proteins by secondary reaction on the nucleophilic side chains of cysteine, histidine, and lysine side chains, with aldehydes produced during lipid peroxidation (Dalle-Donne et al., 2003).

There are many natural sources of oxidative stress, such as UV radiation, heat shock and inflammation. On another hand, there are endogenous sources of ROS in cells, such as oxidizing enzymes (*e.g*. cytochrome P450 that can produce $O_2^{\bullet}$). Additionally, ROS production may increase by cellular exposure to a wide range of toxicants, including organic contaminants and metals (Halliwell and Gutteridge, 1999). At this light, oxidative stress has been extensively considered in any research areas, ranging from aquatic toxicology to biomedical sciences.

Neuronal and glial cells are particularly vulnerable to the attack of ROS, which can eventually lead to neuronal damage and neurodegeneration in several species (review in Uttara et al. 2009). The reason for neuronal cell hypersensitivity towards oxidative stress is related both with anatomic and metabolic factors. For example, glial cells in the brain require more oxygen and glucose consumption to generate the ATP necessary for the normal brain functioning, associated with its demanding activity (in general terms, the brain keep all other organs active and under control). This makes the glial cells more susceptible towards oxygen overload, and thus free radical generation (review in Uttara et al., 2009). Just a small portion of oxygen is converted in ROS, as previously described. However, in an aged brain this percentage goes up related with the reduced surveillance of antioxidants and low regenerative capacity (review in Uttara et al., 2009). Moreover, the brain contains a high level of fatty acids, which are highly susceptible to peroxidation, while not being particularly enriched in antioxidant defenses. Actually, the brain has lower antioxidant activity in comparison with other tissues (*e.g*. it has just 10% of liver antioxidant activity) (review in Uttara et al., 2009).

1.4. Advantages provided by marine macroalgae-enriched feeds to farm fish: emphasis on antioxidant protection and neurotransmission

The use of different MM as a supplementary feed resource in animal production is not recent (Evans and Critchley 2014; Angell et al. 2016; Garcia-Vaquero and Hayes 2016; Makkar et al. 2016). So far, there are only a couple of studies that had investigated the role of MM dietary supplementation on antioxidant responses in fish. Peixoto et al. (2016) had assessed the benefits of MM enriched feeds (a mixture of *Gracilaria*, *Fucus* and *Ulva* in a total percentage of 7.5 %) on the liver antioxidant responses of *Dicentrarchus labrax*, as well as the individual benefits of a *Gracilaria*-supplemented diet. The MM mix diet increased GR activity when compared with fish under a control diet, while the *Gracilaria*-enriched feeds had increased lipid peroxidation together with GST activity. However, Fazio et al. (2016) showed that lipid peroxidation can be influenced by the fish feeding habits. According to these authors, herbivorous fish tend to have a lower lipid peroxidation than carnivores or omnivores fish. This information can possibly indicate that a feed supplemented with MM may reduce the levels of lipid damage. Even because, Magnoni et al. (2017) results, indicating that fish fed with 5 % of *Gracilaria* or Ulva had lower levels of lipid peroxidation when exposed to an acute hypoxia. The alterations in the antioxidant system were observed without a compromise of the fish growth in both tested alternative diets.

The increase of lipid peroxidation in liver of fish feed with *Gracilaria* is intriguing in the way that its suggests that supplementation with this MM increases the degradation of the lipid layer, as described in Peixoto et al. (2016). However, this observation was in accordance with a previous study that revealed a dose-dependent inhibition of the lipid accumulation in cells treated with *Gracilaria verrucosa* extracts (Woo et al., 2013). Overall, Peixoto et al. (2016) had attributed the differences found in antioxidant enzyme activities in seabass fed with a mix diet as the result of a synergistic effect between *Gracilaria*, *Ulva* and *Fucus*, even if this hypothesis remained unclear due to the lack of research on the effect of *Ulva* and *Fucus* on the antioxidant system in fish. Carotenoids that can be found in the MM, showed the ability to change the antioxidant system, through the decrease of SOD activity with increasing of the carotenoid concentration in the diet of *Hyphessobrycon callistu* (Wang et al. 2006).

As detailed in the previous section, the chief enzymes that restrict oxidative damage are the SOD, CAT and peroxidases that convert hydrogen peroxide to water. In eukaryotic algae, the superoxide

dismutase has Mn or Fe as cofactors, or some combination of Fe, Mn and Cu+Zn. Moreover, catalase has a Fe-containing heme cofactor, while peroxidases use a reductant to convert hydrogen peroxide to water. Some of these enzyme cofactors, such as Cu and Zn, and particularly Fe, are used in numerous metabolic pathways (Wells et al., 2016). Since the ingested antioxidant enzymes are digested in the intestine (Figure 6), it is believed that the only effect that the MM antioxidant enzymes can have is through the uptake of the associated metal cofactors across the intestinal epithelium (Wells et al., 2016). Nevertheless, the possible effects on the intestinal microbiome of any undigested enzyme, or of the released metal cofactors, have not been investigated yet. Additionally, MM can be a source of selenium (Schiavon et al., 2017) that is an essential element for Se-requiring glutathione peroxidase. However, the knowledge of the factors regulating Se content of algal foods and its availability to the organisms that consume MM remains elusive. Moreover, algae are composed by a wide variety of molecules capable of scavenging ROS, as observed *in vitro* and *in vivo* cells. These molecules comprise mainly: i) the water-soluble ascorbate (vitamin C); ii) the lipid-soluble α-tocopherol (vitamin E); iii) carotenoids such as astaxanthin; iv) phenolic compounds; v) sulfated polysaccharides (Halliwell and Gutteridge, 1999). Fish can absorb non-enzymatic antioxidants, such as, sterols or peptides present in the MM that in turn may influence the production of enzymatic antioxidants. For example, sterols, such as fucosterol form provided by several brown MM to fish showed antioxidant activity, resulting in increased activities of CAT, GPx or SOD (Kristinsson, 2014). Instead of serving to facilitate the control of ROS, some MM components can inhibit their production, but most studies do not properly distinguish between the decreased production and increased removal of ROS (Wells et al., 2016). There are substantial knowledge gaps on the efficacy of antioxidant properties of MM at several levels, ranging from the characterization among species, through the effects on gut microbiota and transport across the gut lumen to their impacts on fish physiology.

One of the most interesting neurological protective effects of the MM is the influence in the cholinergic activity, as Suganthy et al. (2010) reported the neuroprotective effect of eight different MM (*Enteromorpha intestinalis*, *Dictyota dichotoma*, *Ulva reticulata*, *Gracilaria edulis*, among others). The compounds in the eight MM were capable to inhibit the cholinesterase activity. Hodges (2006) demonstrated that inhibition of AChE plays a key role enhancing the cholinergic neurotransmission in the brain and reduce the aggregation of β-amyloid. DHA is critical for visual acuity, while it is implicated in the activities that underlie cognitive development, such as modulating synaptic efficiency, transmission speed, and myelination processes, as described in section 1.2. Generally, the dietary

sources of DHA to farmed fish are in the form of fish meal and fish oil that are dominant in the compounds feed for fish (Naylor et al., 2000). Marine macroalgae can be an alternative source of DHA to fish, as demonstrated in Kumari et al. (2010). The dietary intake of DHA showed benefits in the neurovascular and neurological health in zebrafish (Sierra et al., 2012). Furthermore, studies with EPA and DHA applied extracellularly raise the stimulatory thresholds of CA1 neurons in hippocampal slices (Xiao and Li, 1999) and the arachidonic acid (ARA) inhibits sodium currents and synaptic transmission (Fraser et al., 1993). Furthermore, DHA treatments in zebrafish can modulate the brain to be more resistance to neurotoxic insults, such as contaminants of pharmaceuticals (Sierra et al., 2012).

The benefits of MM enriched aquafeeds on fish brain and sensory organs were not addressed, yet. Despite that, neuroprotection afforded by MM to fish can be speculated at the light of mammals' findings (details in section 1.1 and Table 1), which is also supported by the homology between fish and mammals regarding the main neurotransmitter pathways and neuroanatomy (section 1.2). Since MM are at the base of the aquatic trophic chains, representing an important natural food source to wild fish, particularly for some species that seem to be well adapted to its consumption (Norambuena et al., 2015). *Sparus aurata* is probably a good example, since in the wild it was found that its diet was composed by small crustaceans and molluscs, however, will also consume algae (Alarcón et al., 2001).

Only a few studies had been focused on the beneficial effects of MM on fish sensory organs and brain. Marine macroalgae had lutein and zeaxanthin, two carotenoids, with beneficial effects (Ma and Lin, 2010). Abdel-Aal et al. (2013) described the existence of this two compounds in the retina cells. Lutein and zeaxanthine are transported into retina in the same ratio that to the plasma, and then transferred to macula where lutein is preferentially converted into meso-zeaxanthine, a non-dietary carotenoid that is not found in the serum, but only in the retina. These evidences suggest the importance of lutein, zeaxanthin and meso-zeaxanthin in the good eyes health. Furthermore, carotenoids present in green and brown MM have a beneficial effect in the fish eyes (Abdel-Aal et al., 2013).

Farmed fish can have smaller brain in relation with their wild conspecifics, as far as was reported for *Oncorhynchus mykiss* (Marchetti and Nevitt, 2003; Kihslinger, 2005). Due to the existence of bioactive compounds in the MM that are vital for the good brain functioning and development, such as DHA and EPA (already described above), it is expected that feed the farm fish with MM enriched-meals to farmed fish could counterbalance this problem.

Figure 6: Schematic illustration of the microbiota–gut–brain axis and how the essential brain nutritional elements and antioxidants are related to the contents of marine macroalgae. AA: Amino acid; B_{12}: Vitamin $B_{12;}$ DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; Mg: Magnesium; Zn: Zink (Adapted from: Cornish et al. (2017)).

The advantages of using MM can also include high growth rate, potential cultivation in saltwater, together with an independence of arable land and industrial fertilization (Øverland et al. 2018). In fact, MM are starting to be used as a novel food ingredient in pisciculture (Batista, 2008) with the main aim of enhancing fish health and aquaculture productivity. Fish aquaculture has been increasing its production, which is followed by the need of improving organism's welfare. The use of MM, under this context, has an added value particularly considering several farming protocols that can severely affect fish physiology, as already documented for the immune system (Queiroz et al., 2014; Peixoto et al., 2016). *In vitro*, all three groups of macroalgae (i.e., red, green and brown) have shown antimicrobial properties and inhibitory effects against fish pathogen (Øverland et al. 2018). Conversely, still there is limited information on the effect of dietary macroalgae supplementation on health of farmed fish *in vivo* (Øverland et al. 2018). Even though, there is an increasing interest on the use of MM as a bioactive component in functional feeds for fish. In this direction, it was reported an improvement

in growth performance and a lower lipid content in the carcass of *Nile tilapia* feed with 5 % of *Ulva* (Ergün et al., 2009). Additionally, previous studies suggested that feed supplemented with MM could mitigate fish stress responses and improve vitality (Mohamed et al., 2012), while increasing illness (Araújo et al. 2016) resistance together with the flesh quality (Valente et al., 2016), representing unquestionable advantages for the aquaculture industry (Luna-Acosta et al., 2011).

1.5. Cultivation and nutrition of gilthead seabream (*Sparus aurata*)

The gilthead seabream, *Sparus aurata* (Figure 7), is a teleost species belonging to the Sparidae family. This species can be found in the Atlantic Ocean, from the British islands, Gibraltar Strait to Cape Verde, around the Canary Islands, and in all the Mediterranean Sea. It is an eminently coastal species, living on rocky or sandy bottoms. Gilthead seabream is a sedentary fish that migrates alone or in small aggregations, moving in early spring towards protected coastal waters, in order to find abundant food and mild temperatures. The feeding habits are based on shellfish (bivalves and gastropods) and crustaceans, although can also feed on small fish and macroalgae (Madeira et al., 2016).

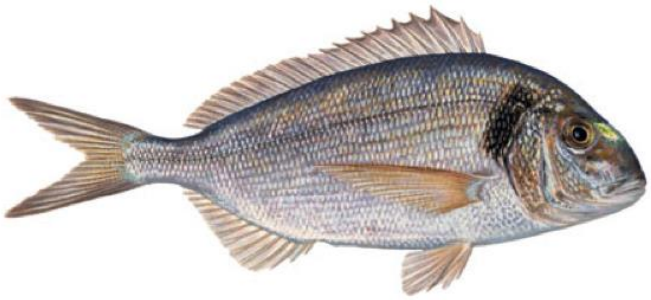

Figure 7: Gilthead seabream *Sparus aurata* (adapted by Desouky and Jover (2016))

The *Sparus aurata* is a very suitable species for aquaculture in the Mediterranean region, as well as in Portugal, due to their good market price, high survival rate and feeding habits (which are relatively low in the food chain), as well as due to the fact that it is possible to control their whole life in captivity (FAO, 2004). In the early years of farm fish, gilthead seabream was traditionally cultured extensively in coastal lagoons and saltwater ponds (FAO, 2004). In this type of systems, it is generally reared with mullets, seabass or ells and feed naturally. However, this species can also be reared in the semi-intensive or intensive systems. In semi-intensive conditions, the natural diet is supplemented with

a commercial feed. In fact, the commercial food makes it possible the creation of polycultures. Furthermore, *Sparus aurata*, are widely used in Portugal as an accessorily herbivorous in polyculture system with european seabass (*Dicentrarchus labrax*), in order to prevent the excessive growth of macroalgae in the pounds, a frequent problem in seabass monoculture pounds (Afonso, 2016). In the intensive systems, gilthead seabream is fed exclusively with commercial pellets (European commission, 2012). *Sparus aurata* has been highly farmed in Europe, being its production growing globally (FAO, 2015) (Figure 8).

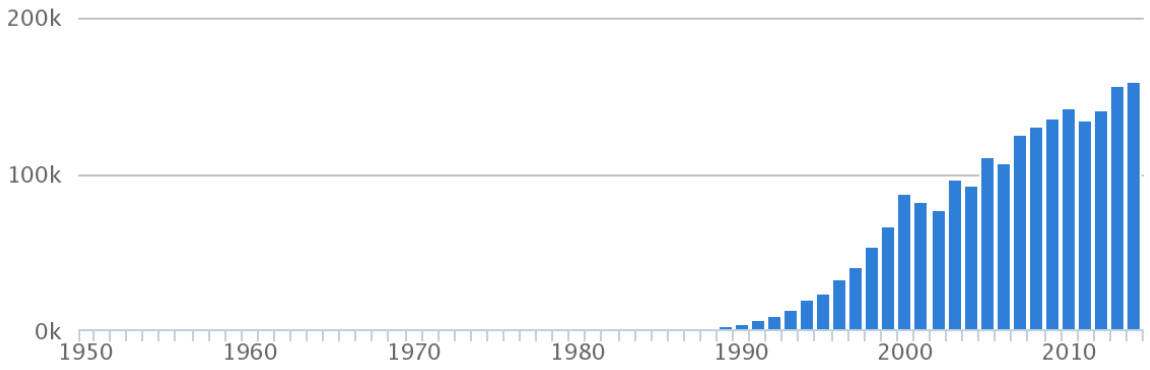

Figure 8: Global aquaculture production of *Sparus aurata* (tonnes) (Adapted from: FAO FishStat cited in FAO, 2015).

1.6. Potential hazards to fish in aquaculture: the formalin case

It has been estimated that fisheries and aquaculture supplied the world with around 110 million metric tons of food fish per year (FAO, 2010). Aquaculture production supports 47% of this supply, with intensive aquaculture gaining a chief importance to meet the population needs. Intensive aquaculture is a system characterized by a high density of production of aquatic species (level of production up to 200 tonnes ha^{-1} $year^{-1}$), with a high level of control, technology and high production efficiency and nutritionally complete feeding, using man-made culture systems (FAO, 1988). Maintaining the high level of production is highly demanding, which had led to the use of several chemicals along the process. In fact, there is a variety of chemicals that are frequently used in the aquaculture production of fish, including: (i) disinfectants (*e.g.*, hydrogen peroxide, malachite green, formalin); (ii) antibiotics

(*e.g.*, sulfonamides and tetracyclines); (iii) anthelmintic agents (*e.g.*, pyrethroid insecticides and avermectins) (Hossain et al., 2013).

The intensive production of fish may occur in open circuits or in closed or semi-closed circuits. Particularly, closed or semi-closed systems are a vulnerable to pathogens occurrence, and then to the spreading of diseases (Yanong, 2003). Bacteria, fungi, viruses and parasites can be considered pathogen agents, since are potentially disease-causing organisms. Depending on the infectious agent, clinical signs can vary from a minor skin rash in just a reduced number of fish to a quick outbreak of disease, having all fish stock with reduced appetite, lethargy and eventually heavy losses (Shepherd and Bromage, 2001). In closed or semi-closed systems there are several potential spots of pathogen concentration, namely the mechanical filter and the equipment, which can be transmitted by the water, fish to fish and fomites (inanimate objects that can transmit diseases) (Yanong, 2003). In intensive fish production, infectious diseases can have dramatic consequences, especially in high stock situations, and therefore the best way to control the diseases is working on prevention. The disinfection practice is particularly relevant to minimize pathogens of spreading, generally involving the use of some chemicals, namely: ethyl alcohol; copper sulphate; iodine, potassium permanganate; formalin; hydrogen peroxide. Formalin is one of the most applied disinfectants in intensive aquaculture (Boyd and McNevin 2015). It may be used as a prophylactic measure or with therapeutic purposes and it is extremely effective against most protozoan parasites (*Ichthyophthirius* spp., *Costia* spp., *Epistylis* spp., *Chilodonella* spp., *Scyphidia* sp., *Trichodina* spp.) and trematodes (*Cleidodiscus* spp., *Gyrodactylus* spp. and *Dactylogyrus* spp.) (Francis-Floyd 1996; Shao 2001; Leal et al. 2016).

The commercial formulation of formalin is an aqueous solution, containing usually 37–40% by weight of formaldehyde gas per weight of water (Leal et al., 2016). The aqueous solution of formaldehyde is stabilized with 10–15% of methanol by weight to prevent the formation of paraformaldehyde that is very toxic to fish (Kitchens et al., 1976). The use of methanol as stabilizer of the formalin solution can raise some concern related to the toxicity of that compound to fish. In fact, it was reported fish hyperactivity and convulsion, as well as respiratory distress, reduction in growth and impairment of reproductive performance in fish exposed to formalin (Kaviraj et al., 2004). LC_{50} is an indicator of acute toxicity and correspond to the concentration able to kill 50 % of the exposed organisms (Rand, 1995). Studies pointed out that methanol LC_{50} (at 96 h) ranges between 15.400 and 29.400 mg L^{-1} to fish (Kaviraj et al., 2004). Furthermore, the methanol LC_{50} are not the only LC_{50} that should be considered in the formalin administration. The median lethal concentrations (LC50) of

formalin can vary according to the exposure duration, water temperature and between species. For example, the LC_{50} for olive flounder fingerlings (*Paralichthys olivaceus*) is 209 mg L^{-1} during 24 h with 12 ºC or 182 mg L^{-1} during 48 h with 12 ºC of water temperature. The juvenile of striped bass (*Morone saxatilis*) showed a LC_{50} of 455 mg L^{-1} during 6 h with 17 ºC of water temperature or 48 mg L^{-1} during 96 h with 17 ºC.

In intensive aquaculture, formalin is frequently added to water and the concentration applied for treatment should be sufficient to kill the infectious agent, but without endangering the fish. The treatment should be performed very carefully and only when it is necessary, but the gap may be small between the concentrations needed to kill the infectious agent and the preservation of the fish health (Shepherd and Bromage 2001; Leal et al., 2016). For an appropriate treatment, important factors such as water flow rates, operating volume of the tank, temperature and oxygen levels should be considered and, the concentration applied to the treatment is strongly dependent of the fish age (Leal et al, 2016).

The observation of fish behavior needs to be ensured during the treatments, which can be short-term bath or a prolonged immersion in formalin (prolonged bath). The most common dosage of formalin for the short bath treatment is up to 250 mg L^{-1} up to 1 hour (commonly varies between 30 and 60 min) (Shepherd and Bromage, 2001). Considering a solution of formalin of 250 mg L^{-1} containing 37% by weight of formaldehyde gas per weight of water, the conversion of formalin concentration to formaldehyde concentration is achieved by multiplying 0.37 by 250 mg L^{-1} (formalin concentration), which is equal to 92.5 mg L^{-1} (formaldehyde concentration) (Leal et al., 2016). Due to the electrophilic character of formaldehyde hydrate (formalin), it is able to react with functional groups of several biological macromolecules, namely with proteins, DNA and RNA, polysaccharides and glycoproteins. Reactions of formalin with functional groups seems to occur by the intra- and inter-molecular cross-linking of macromolecules that modify the physical characteristics of tissues (Fox et al., 1985; McDonnell and Russell, 1999; Leal et al., 2016). The formalin reaction with proteins or with DNA and RNA in order to inactivate microorganisms occur mainly by reaction with amino acids and nucleic acids, respectively (Leal et al., 2016). The alkylation of ring nitrogen atoms of purine bases of the nucleic acids is the action way proposed, as exemplified in the reaction for adenine in the figure 9. However, the reaction with deoxynucleotides is pH dependent and occurs rapidly.

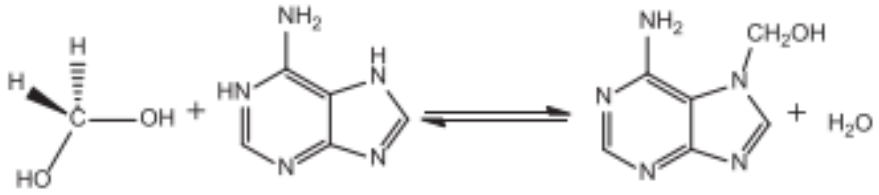

Figure 9: Reaction between the formalin and the adenine base (adapted from Leal et al, 2016).

Regarding to the formalin interaction with proteins, different reactions such as denaturation (Maris, 1995) and alkylation of amino and sulfhydryl groups are proposed in the literature. Furthermore, the formalin reacts particularly with the α-amino acid groups and in some cases with the side chains groups of amino acids (Kiernan, 2000).

There are some studies where fish exposure to formalin/formaldehyde was followed by toxic effects, but there are also other works where no toxicity was reported. Leal et al. (2016) had made a recent revision of formalin/formaldehyde toxicity in fish, and therefore some of the findings already mentioned in that article are summarized here. For instance, some authors had observed a permanent damage of the gills (Shepherd and Bromage, 2001) while others reported alterations in the mucous cells of the caudal fin (Buchmann et al., 2004). A reduction of the blood pH and an increase of the plasma protein concentrations were also reported for the rainbow trout and Atlantic salmon (WHO, 1989). Jung et al., (2003) performed several biochemical tests in olive flounder (*Paralichthys olivaceus*) and observed that most part of the analyzed parameters is significantly modulated by the presence of formalin in a range of concentrations of 100 – 300 mg L^{-1}. However, these results are obtained in the end of 3h and not in the end of the recommended exposure time (1h). In fish, Ispir et al. (2017) showed that the acute exposure to formalin in concentrations of 100 µg/mL and 200 µg/mL cause physiological alterations in the antioxidant system (CAT, SOD, GPx) in whole body of *Oncorhynchus mykiss*. Studies in *Sparus aurata* or in the *Dicentrarchus labrax* shows high levels of plasma cortisol and disrupted hydromineral balance when individuals of both species were exposed to 150 ppm of formalin during 1h (Yildiz and Ergonul, 2010).

Contrarily, no effects were observed on the gill structure of juvenile salmonids after repeated prophylactic formalin treatments (167–250 mg L^{-1}) (Speare et al., 1997), was only noticed an increase of the mucous cells' number on gill lamellae (Speare et al., 1997). Accordingly with Chinabut et al.

(1988), continuously exposure to formalin (25, 50 and 75 mg L^{-1}) by common carp during 8 weeks did not confer histological changes in gills, liver, kidney, spleen, intestine, muscle and skin. Overall, the toxic effects of formalin/formaldehyde seem to be dependent on the dose, fish species, exposure conditions, and water physical-chemical features, as discussed by Leal et al. (2016). Another relevant aspect of formalin toxicity is that formaldehyde is highly soluble in water and its bioaccumulation in aquatic organisms is presumably low, due to its low n-octanol/water partition coefficient (WHO, 1989).

So far, no studies were focused on the neuronal and sensory effects of formalin/formaldehyde in fish. The fish eye is particularly vulnerable to formalin exposure, considering the administration mode. Moreover, effects on the gills mucus of juvenile salmonids were already reported, as detailed. The fish eyes are covered by a mucus, with the similar function that the mucus in the gills (Zamzow, 2004). When the eyes and gills are exposed to a contaminant, the mucous cells acts as a first line of defense through the mucous segregation (Coello and Khan, 1996). Due to this and the fact that both tissues are in permanent contact with the aqueous medium, it is expected effects in the eyes, similarly to those obtain in the gills.

However, neurotoxic effects in mammals, particular in humans and monkeys were reported (Songur et al. 2010). The formaldehyde was observed to affect the cerebral antioxidant system, causing oxidative damage in the humans (Songur et al. 2010). The human exposure to 10 mg/Kg during 10 days showed significant differences in the oxidant substances, such as increase in protein carbonyl and malondialdehyde or a decrease in the activity of antioxidant enzymes, such as SOD and CAT in the rat fontal cortex and hippocampus (Gurel et al., 2005). Formalin is also mutagenic, carcinogenic and when injected can lead to several disorders of the central nervous system or can provoke necrosis in the brain (Pandey et al., 2000). In other organisms, formalin had led to an inhibition of AChE activity in earthworm *Eisenia andrei* (Hackenberger et al., 2012). Although, the cutaneous administration of low concentrations of formalin can initiate a temporary over-adaptation that led to an increase of AChE activity in the *Eisenia andrei* (Hackenberger et al., 2012). Furthermore, alterations in the brain may lead to alterations in the fish behavior or in the capacity to adapt to unfavorable conditions.

The blood-retinal-barrier and blood-brain-barrier are natural barriers presented in the eyes and brain structure, respectively (Mills et al., 2010; Tomi and Hosoya, 2010). These barriers between the blood and the retinal or brain tissues can confer some kind of protection against the xenobiotic, due to the impermeability capacity to the external compound (Pereira et al. 2015). Due to this, it can be expected an active protection against the formalin exposure in the brain tissue. However, due to the

formalin administration path (through the water), the retinal-blood-barrier may not confer the same type of protection.

1.7. Thesis aims and structure

Overall, there is a poor knowledge on the protective effects afforded by marine macroalgae (MM) to farmed fish eyes and brain, particularly related to the antioxidant protection and neurotransmission. This study was designed as a contribution to overcome this research gap, by investigating, for the first time, the antioxidant and neurotransmission protection afforded by a MM-enriched diet to the eyes and brain of the gilthead seabream (*Sparus aurata*). Under this aim, the fish where fed with MM-enriched feeds (total incorporation of 5%, with the species, *Fucus vesiculosus*, *Gracilaria gracilis* and *Ulva rigida* equitably represented) for 2 months to establish a different dietary background to that of non-supplemented fish (feed with a standard diet, without macroalgae). Then, in order to evaluate the protection afforded by a macroalgae-enriched diet, fish were exposed to a common disinfectant agent in aquaculture (formalin) that would possibly act as an exogenous challenge to fish. This hypothesis was supported in the literature that reported effects of formalin/formaldehyde in fish under conditions of prophylactic administration, as considered here. The antioxidant and neurotransmission protection afforded by macroalgae-enriched diet to the eyes and brain of the gilthead seabream (*Sparus aurata*) upon formalin exposure was investigated by analysing two sets of endpoints, namely: (i) enzymatic and non-enzymatic antioxidants, as well as damage indicators; (ii) acetylcholinesterase as a paradigmatic endpoint of the neurotransmission condition. As an overarching aim, this study will bring new information that can improve the fish welfare in aquaculture, particularly when submitted to a commonly used compound that could highly challenge fish health condition.

2. MATERIALS AND METHODS

2.1. Experimental design

The experiment was performed with gilthead seabream (*Sparus aurata*) provided by the company Nasharyba, produção e comércio de peixe, LDA. Fish were held in 10 tanks of 230 L for three weeks for acclimation (Figure 10). The initial fish weight was 21.5 ± 4.9 g and the initial fish length was 11.1 ± 1.1 cm. Then, during 2 months fish were fed with marine macroalgae (MM) enriched feeds [total incorporation of 5%, with the species *Fucus vesiculosus*, *Ulva rigida* and *Gracilaria gracilis*, equitably represented, corresponding to the MM supplemented fish (group A)], while non-supplemented fish were fed with a standard diet (fish group S - feed without MM). During this 2 months period, the dietary background was settled (Figure 10). After that, both dietary groups were subjected to a formalin (F) short-term bath, consisting in fish immersion over one hour (10 fish at a time) in an tank of 100 L of seawater spiked with a formalin solution ($CH_2(OH)_2$) (formaldehyde solution 37 % (CH_2O) provided by Merck (CAS:50-00-0) stabilized with 10 % of methanol), diluted 45 % to reach a final concentration of 150 µl L^{-1}.The same treatment with formalin was repeated two days later. Control groups, unexposed to formalin, were maintained over the experiment (fish groups A and S). In order to simulate the conditions of formalin exposure, the water level of the tanks A and S has been lowered to the level of the tank of exposure (100 L) and the water was shaken to simulate the fish handling. Four and 18 days after the 1st exposure to formalin, fish of the different 4 groups (i.e. S, A, SF, AF) were sacrificed, upon anesthesia with tricaine methanesulfonate (MS-222), weighed and measured (Table 2). Fish were sacrificed by cervical transection and then were bled (with heparinized Pasteur pipettes at the cardinal vein) and the eyes and brain were removed and stored at −80 °C until further analyses. Eyes and brain samples were collected for antioxidants analyses, namely for: i) catalase (CAT); superoxide dismutase (SOD); total glutathione (GSHt); glutathione peroxidase (GPx); glutathione S-transferase (GST); glutathione reductase (GR, on eyes only). Damage indicators were also assessed [lipid peroxidation (LPO) and protein oxidation (PO, only for eyes)], as well as the activity of acetylcholinesterase (AChE). During the whole experiment, fish were fed twice a day, either with MM-enriched feeds or with the standard diet. Water physical-chemical parameters and water flux were monitored daily, over the experiment, in the tanks where fish under a standard diet (S) and a MM-enriched (A) diet. Parameters varied as follows for: tank S: temperature: 20 - 22 ºC; dissolved oxygen: 5.0 - 5.6 mg L^{-1}; pH: 7.4 - 7.9;

water flux: 0.01 L s^{-1}; tank A: temperature: 20 - 22 ºC; dissolved oxygen: 5.2 - 6.1 mg L^{-1}; pH: 6.8 - 7.5; water flux: 0.01 L s^{-1}.

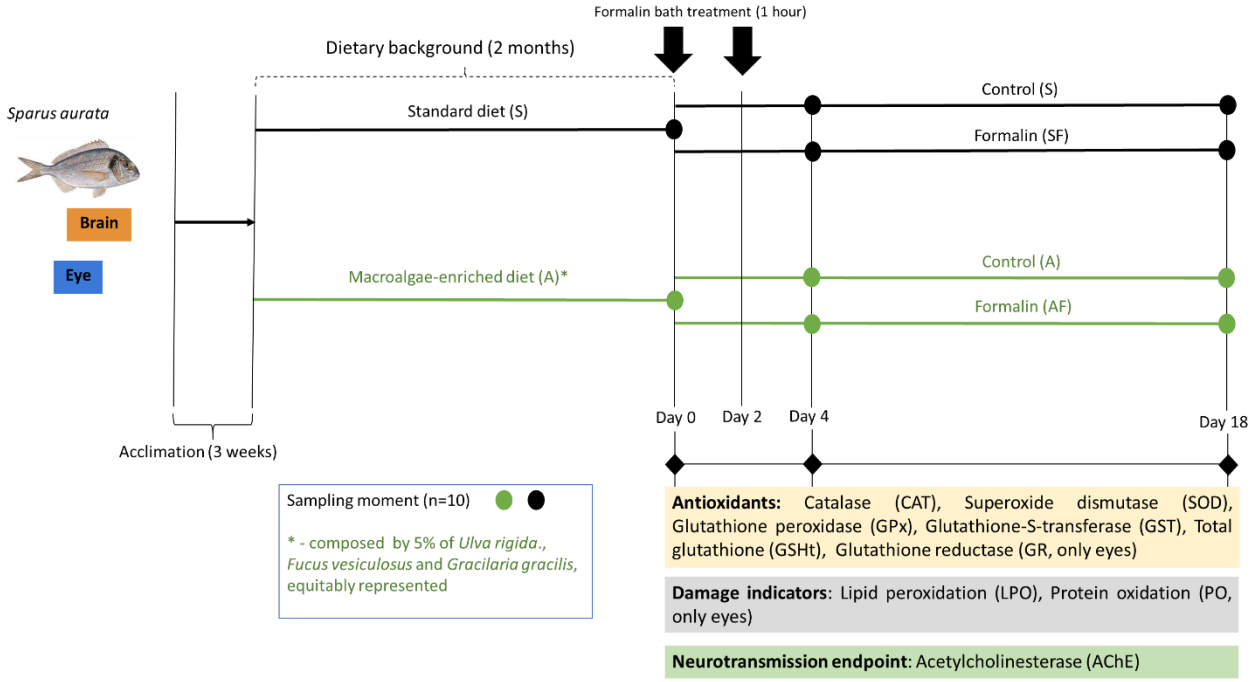

Figure 10: Design of the experiment with gilthead seabream (*Sparus aurata*). Prior to formalin exposure, the fish were acclimatized during 3 weeks and after that the fish were divided in two groups and fed with a standard diet (S) and a macroalgae-enriched diet (A) during 2 months (dietary background settlement). The fish were exposed in two separated times, in the end of 2 month and 2 days later. Thereafter, the fish eyes and brain of the four treatments (S, SF, A, AF) are collect 4 and 18 days after the first formalin exposure. Eyes and brain samples were collected for antioxidants analyses, damage indicators and for the determination of acetylcholinesterase (AChE) activity.

2.2. Biochemical analyses in the eyes and brain

The eyes tissue were homogenized in a 1:5 ratio (eyes weight:buffer volume) and brain tissue in a 1:8 ratio (brain weight:buffer volume) of chilled phosphate buffer (0.1 M, pH 7.4) using a Potter-Elvehjem homogenizer. The homogenate was divided in two aliquots, one for lipid peroxidation (LPO) analyses and another for the isolation of the post-microsomal supernatant (PMS) preparation (Figure 11). The aliquot for LPO evaluation was stored with 1:10 butylated hydroxytoluene (prepared in 4 % of methanol) and phosphate buffer. The PMS fraction, obtained by centrifugation in a refrigerated

centrifuge (Eppendorf 5415R) at 13,000 g for 20 min at 4 °C, was divided in aliquots to be used for different antioxidant determinations, oxidative damage and the measure of AChE activity.

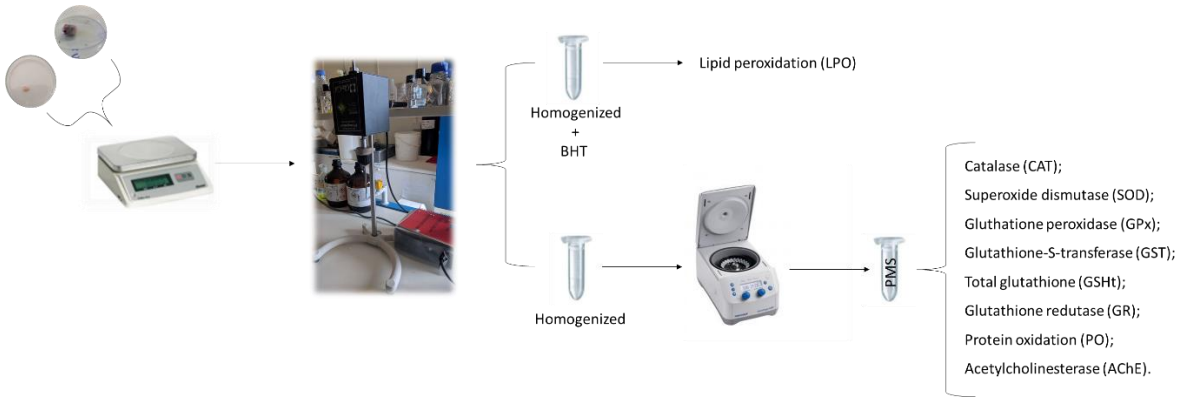

Figure 11: Schematization of the sample preparation procedure for further biochemical quantification**.** PMS: post-microsomal supernatant**.**

All the aliquots were stored at −80 °C until spectrophotometric analyses in a SpectraMax 190 microplate reader (at 25 °C), which consisted on the following procedures:

- Catalase (CAT) activity was measured in PMS following the Claiborne (1985) method as described by (Giri et al., 1996). Briefly, the assay mixture differed slightly between brain and eyes PMS. For the brain it was used 190 µL of hydrogen peroxide (10 mM) and 10 µL of sample, while for the eyes 195 µL of hydrogen peroxide (10 mM) and 5 µL of sample, reaching in both cases a final volume of 200 µL. Change in absorbance was recorded at 240 nm and CAT activity was expressed in terms of mmol per min per mg of protein per min, using a molar extinction coefficient (ε) of 43.5 M^{-1} cm^{-1}.

- **Superoxide dismutase (SOD)** activity was measured in PMS using a spectrophotometric enzymatic kit (RANSOD TM, Randox) following manufacturers' instructions, and adapted to microplate. This methodology employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-ni-trophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. Changes in absorbance were recorded in 30 s cycles for 3 min at 505 nm. SOD activity was then measured by the degree of inhibition of this reaction. One unit of SOD is the amount that causes a 50 % inhibition of the rate of reduction of INT, under the conditions of the assay. Results were expressed as SOD units per mg of protein.

- **Glutathione peroxidase (GPx)** activity was determined in PMS according to the method described by Flohé and Günzler (1984), adapted to 96-well microplate. The assay mixture consisted of 90 µL phosphate buffer (0.05 M, pH 7.0), 30 µL of PMS (diluted properly), 30 µL GR (2.4 IU mL^{-1}), 30 µL reduced glutathione (GSH; 10 mM), 30 µL sodium azide (10 mM), 30 µL EDTA (10 mM), 30 µL NADPH (1.5 mM) and 30 µL H_2O_2 (2.5 mM) and in a total volume of 300 µL. GPx activity was determined by monitoring the oxidation of NADPH to $NADP^+$, resulting in an absorbance decrease at 340 nm. The absorbance was read every 30 s for a period of 5 min. GPx activity was calculated in terms of nmol NADPH oxidized per min per mg of protein, using a ε of 6.22 mM^{-1} cm^{-1}.

- **Glutathione-S-transferase (GST)** activity was determined according to the method of Buczyński et al. (2006) using CDNB (1-chloro-2,4-di-nitrobenzene) as substrate. The assay was carried out in a 96-well microplate with a 100 µL of PMS (diluted properly) and 175 µL of GSH (1.765 mM; prepared in phosphate buffer 0.2 M, pH 7.9). The reaction was initiated by addition of 30 µL of 1-chloro-2,4-dini-trobenzene (CDNB; 10 mM), and the increase in absorbance was recorded spectrophotometrically at 340 nm, during 5 min each 30 s. Glutathione-S-transferase activity was expressed as nmol of thioesther produced per min per mg of protein, using a ε of 9.6 mM^{-1} cm^{-1}.

- **Total glutathione (GSHt)** content was measured following the method of Baker et al. (1990) adapted to a microplate reader by (Vandeputte et al., 1994). Protein content in the PMS was precipitated with trichloroacetic acid (TCA 12 %) for 1 h and then centrifuged at 12,000 g for 5 min at 4 °C. Total glutathione was determined (in deproteinated PMS) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) producing a yellow colored 5-thio-2-nitrobenzoicacid (TNB). Reaction mixture containing 1 mM DTNB, 0.34 mM NADPH dissolved in a stock sodium phosphate buffer (143 mM with 6.3 mM EDTA, pH 7.4) was added to wells containing 40 µL of deproteinated PMS (previously diluted 1:3) and the reaction was started by adding 40 µL of 8.5 IU mL^{-1} GR. Formation of TNB was monitored by spectrophotometry at 415 nm for 7 min. The results were expressed as nmol TNB conjugated per min per mg of protein, using a ε of 14.1 mM^{-1} cm^{-1}.

- **Glutathione reductase (GR)** activity was assayed by the method of Cribb et al. (1989) adapted to 96-well microplate. Briefly, the assay mixture contained 50 µL of PMS fraction and 250 µL of NADPH (0.206 mM), glutathione disulfide (GSSG - 1.068 mM) and diethylene triaminepentaacetic acid (DTPA - 0.549 mM). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm during

5 min. The enzyme activity was calculated as nmol NADPH oxidized per min per mg of protein, using a ε of 6.22mM^{-1} cm^{-1}.

- **Lipid peroxidation (LPO)** was determined in the previously prepared homogenate as adapted by Wilhelm Filho et al. (2001) after Bird and Draper (1984). Briefly, 250 µL of TCA (12 %) in aqueous solution, 225 µL of Tris–HCl (60 mM, pH 7.4, and 0.1mM DTPA) and 250 µL of TBA (0.73 %) were added and thoroughly mixed with 150 µL of the homogenate. This mixture was heated for 1 h in a water bath set at boiling temperature and then cooled to room temperature, decanted into 1.5 mL microtubes and centrifuged at 15,800g for 5 min. Absorbance was measured at 535 nm, and LPO was expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) formed per mg of protein, using a ε of 1.56×105 M^{-1} cm^{-1}.

- **Protein oxidation (PO)** were determined as a measure of protein oxidation, using a commercial kit (Protein Carbonyl Content Assay Kit – Sigma Aldrich), according to manufacturer's instructions with some modifications, namely an additional rinse of the pellet with acetone and an additional centrifugation at the final of assay before the reading. The carbonyl content was determined by the derivatization of protein carbonyl groups with 2,4-dini- trophenylhydrazine (DNPH), leading to the formation of stable di- nitrophenyl (DNP) hydrazone adducts, which can be detected spectrophotometrically at 375 nm. Values were expressed as micromoles of DNP hydrazone adducts formed per mg of protein, using a ε of 22 mM^{-1} cm^{-1}.

- **Acetylcholinesterase activity (AChE)** was determined following the method of Ellman et al. (1961) adapted to microplate. Briefly, the reaction medium consisted in 0.096 M of phosphate buffer (pH 7.2), DTNB (0.32 mM), sodium bicarbonate ($NaHCO_3$) (0.57 mM) and acetylcholine 0.48 mM. In a 96-well microplate, 250 mL of reaction medium was added to 50 mL of PMS (brain PMS was previously diluted in phosphate buffer, pH: 7.4 in the proportion 1:5, while the eyes PMS was used directly) (incubate for 10 min before start reading). Formation of thiocholine-DTNB complex (yellow colour) was monitored at 412 nm, for 5 min using a SpectraMax 190 microplate reader. The enzyme activity was expressed as nanomoles of thiocholine-DTNB complex generated min^{-1} mg^{-1} of protein using a ε of 13.6 mM^{-1} cm^{-1}.

Protein concentrations in the PMS and homogenates were determined (at 550 nm) according to the Biuret method described in Gornall et al. (1949), upon adjustment to microplate, in order to express enzymatic activities, GSH, TBARS and AChE as a function of protein content. In turn, protein concentration of samples in the carbonyl groups assay was determined at 562 nm, using the BCA

(Bicinchoninic Acid Kit) Assay Kit (Ref – BCA1 AND B9643) according to the manufacturer's instructions. In both methods, bovine serum albumin was used as standard.

2.3. Condition index estimation

The condition index (K) of fish were estimated by the Froese (2006) , according to the following the formula:

$$K = 100 * \frac{W}{L^3}$$

Where:

K= Condition factor;

W= Fish weight (g);

L= Total length (cm).

2.4. Statistical analysis

The statistical analyses were performed with Rstudio software, version 1.0.1 developed by Rstudio, Inc. For testing the differences in the condition factor (K value) between the different treatments at dietary background, 4 and 18 days, a Mann-Whitney U test was applied under the null hypotheses that didn't exist differences between each treatment. Data on antioxidants (both enzymatic and non-enzymatic) did not meet the normality and homogeneity assumptions, even after several attempts of logarithmic, linear and square root transformation. Therefore, to test the differences between the A and S treatments corresponding to the fish in the "dietary background" period, a Mann-Whitney U test was applied under the null hypotheses that S and A treatments do not differed significantly. In order to compare statistically data of the four treatments (S, SF, A, AF) at 4 and 18 days, a Kruskall-Wallis one-way analyses of variance followed by post-hoc Dunn test was performed assuming the null hypothesis that differences between each treatment do not occur at any of the experimental times. Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. Gross fish health condition assessment

No fish mortality was observed during the whole experiment. Though feeding behavior was not strictly monitored, no alterations were perceptible on fish feeding behavior along the experiment, specifically after the formalin exposure. Fish did not differ significantly in weight and length along the experiment. Moreover, no significant differences were found between treatments for fish condition factor (K) values (Table 2).

Table 2: Mean values (± standard error) of weight, length and estimated condition factor (K) in *Sparus aurata* at the different sampling moments (i.e. dietary background corresponds to a sampling after 2 months of macroalgae dietary supplementation; 4 and 18 days corresponds to the days after the first formalin bath). S represents the standard feed; A indicates a macroalgae-enriched feed; experimental groups submitted to formalin treatment are identified by abbreviations with the "F" letter. The grey color indicates the treatments with the standard diet and the green color indicates the treatments with macroalgae-enriched feed.

	Treatment	Weight (g)	Length (cm)	Condition factor (K)
Dietary background	S	72.6 ± 3.9	16.7 ± 0.3	1.6 ± 0.1
	A	70.7 ± 3.1	16.7 ± 0.2	1.7 ± 0.1
4 Days	S	72.5 ± 4.1	16.6 ± 0.3	1.6 ± 0.1
	SF	70.5 ± 4.5	16.5 ± 0.6	1.5 ± 0.2
	A	76.3 ± 3.5	16.8 ± 0.2	1.6 ± 0.1
	AF	69.5 ± 1.7	16.6 ± 0.2	1.5 ± 0.2
18 Days	S	86.3 ± 5.1	17.7 ± 0.4	1.6 ± 0.2
	SF	91.6 ± 4.3	17.9 ± 0.3	1.6 ± 0.1
	A	84.2 ± 5.2	17.7 ± 0.7	1.5 ± 0.1
	AF	87.2 ± 5.4	18.1 ± 0.4	1.5 ± 0.2

3.2. Enzymatic and non-enzymatic antioxidants in the eyes

After the 2 months of dietary background settlement it was recorded a decrease of CAT and SOD activities in fish supplemented with a mixture of marine macroalgae (AF). Differently, GPx displayed a significant increase in fish fed with MM supplementation (AF) after the same 2 months. Later, no differences were recorded in CAT activity between the two different diets, both in formalin exposed-fish and controls (S, A, SF, AF). But, it was still notorious a reduction of SOD activity in the fish' eyes of individuals that were fed with a MM enriched diet (both in fish exposed and non-exposed to formalin,

respectively A and AF). Contrarily to what was recorded upon the dietary background settlement, the GPx activity in fish fed with MM (A) had decreased when compared with the non-supplemented individuals (S). Accordingly, GST showed an activity decrease in fish supplemented with MM that were not exposed to the formalin challenge (A), when compared to the homologous treatment (S). No differences were recorded 4 days after formalin exposure regarding GSHt and GR activities between the two diets, either for fish exposed to formalin or control individuals (SF and AF). Eighteen days after the formalin bath, it was found a consistent increase of the antioxidant defense system in control fish fed with MM (A), as perceived by an increase of the activities of CAT, SOD, GPx and GST in comparison with fish under a standard diet (S). Throughout the experiment, no significant differences were found for the GSHt levels between fish fed with an enriched MM-diet and those under a standard died, either for formalin exposed fish and control individuals (S, A, SF, AF). Interestingly, there was a poor variation of the antioxidants upon formalin exposure (4 and 18 days), between fish under a MM-enriched diet and a standard one. In fact, a couple of differences were recorded, namely for GR that increased significantly in the eyes of fish fed with MM when compared with non-supplemented fish after 18 days of formalin exposure. Additionally, SOD decreased significantly in the eyes of fish exposed to formalin when fed with a MM-enriched diet (AF) relatively to their homologous (SF).

The effect of formalin in the eyes was notorious at the end of 4 days of exposure. Formalin was able to decrease GPx and GST activities in the eyes of non-supplemented fish with MM (SF). This reduction was not counteracted by the MM supplementation (AF), since fish fed with this diet showed an activity decrease (both of GPx and GST), as well. Formalin treatment did not produce significant differences in the GSHt levels in the eyes of non-supplemented fish (SF), but it was noticed a significant increase in the fish fed with MM supplementation (AF). On the other hand, formalin induced an increase of GR activity in eyes of non-supplemented fish (SF), while MM-enriched diet probably prevented this increase in supplemented fish (AF). Formalin did not induce changes of CAT and SOD activities, in fish under both diets (SF and AF). Eighteen days after the formalin exposure, the activity of CAT increased significantly in fish under a standard diet (SF), while a decrease was recorded in fish under MM-supplementation (AF). Furthermore, the formalin induced a decrease of SOD activity in the eyes of the supplemented fish (AF), which was not found in non-supplemented individuals (SF). At this time, formalin was on the basis of important variations of the glutathione cycle in eyes, as indicated by an increase of the GST activity and a decrease in the GSHt levels in the non-supplemented fish (SF). Both changes were counterbalanced by the MM-enriched feed (AF), as no variations were found.

Furthermore, formalin induced an increase in the GR activity in the eyes of MM supplement fish (AF), which was not recorded in fish under a standard diet (SF).

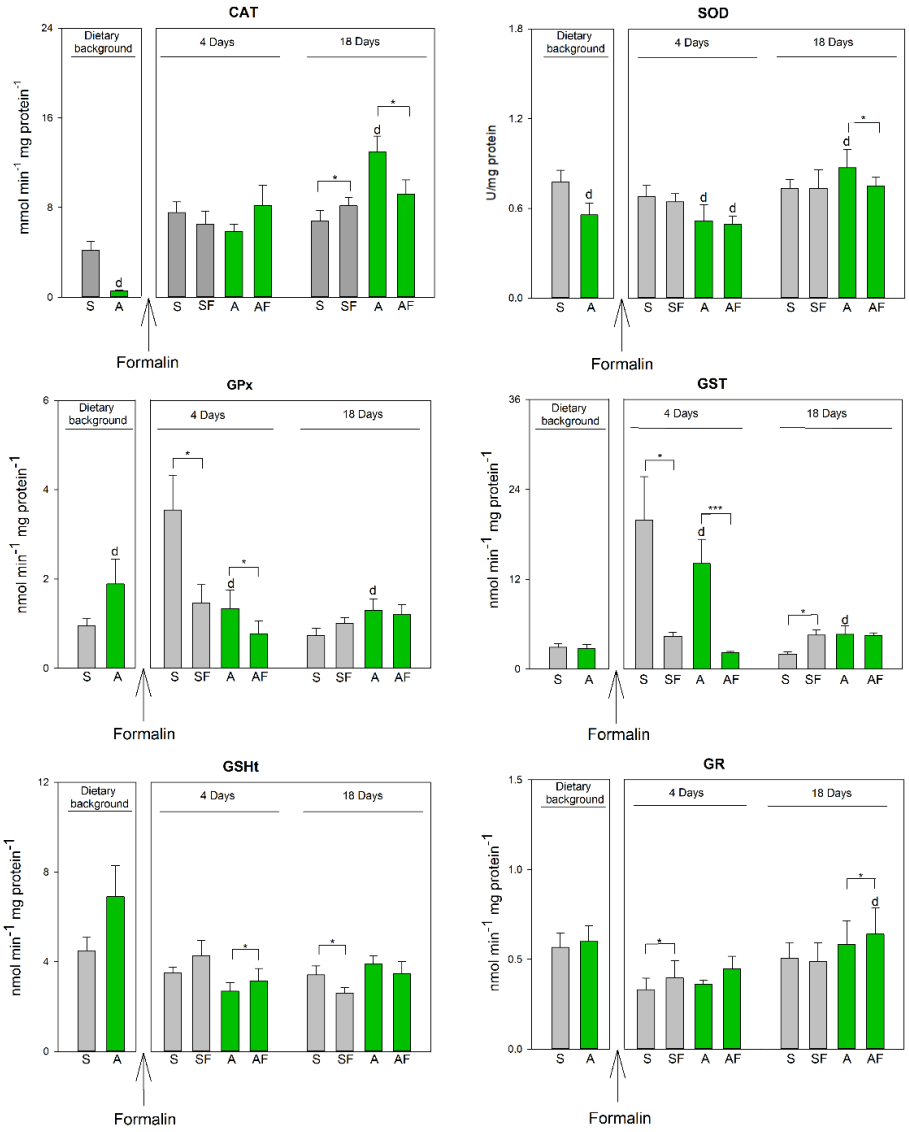

Figure 12: Activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST) and glutathione reductase (GR), together with the levels of total glutathione (GSHt), in the eyes of gilthead seabream (*Sparus aurata*) following 2 months of macroalgae dietary supplementation, as well as 4 and 18 days after a subsequent formalin treatment. Algae-supplemented groups (A) correspond to green bars, while groups fed with standard feeds (S) correspond to gray bars. Experimental groups submitted to formalin treatment are identified by abbreviations with an "F" after the letter identifying the dietary profile (i.e. SF and AF). The grey color represents the standard diet and the green color represents the macroalgae-enriched diet. Data were represented as mean ± standard error. Statistically significant differences between fish exposed to formalin and the respective unexposed group (for each feeding groups) are indicated by * ($p < 0.05$), *** ($p < 0.001$). Statistically significant differences between different dietary backgrounds (within formalin exposed fish and non-exposed fish) are identified by "d" ($p < 0.05$).

3.3. Damage of lipids and proteins in the eyes

After 2 months of dietary supplementation with MM, the baseline values of lipid peroxidation and protein oxidation in the eyes were not significantly different from that recorded for non-supplemented fish. Four days upon formalin exposure, a significant increase of the lipid peroxidation was recorded in the eyes of control fish (unexposed to formalin) supplemented with MM (A) in comparison to non-supplemented fish (S), while no changes were recorded in formalin-exposed individuals (SF and AF). Accordingly, a higher protein oxidation was found in the same control fish supplemented with MM (A), even if a significant decrease was recorded in fish exposed to formalin and supplemented with MM. Eighteen days after formalin exposure, the control fish fed with a MM-enriched diet (A) presented a significant increase in the eyes protein oxidation in relation to fish under a standard diet (S), while no differences were recorded for LPO.

No effects of formalin were perceived in the fish eyes after 4 days of exposure, as far as assessed by LPO. Contrastingly, formalin promoted damage on eyes proteins in fish under a standard diet (SF), which was attenuated in fish supplemented with MM (AF) (protein oxidation was even lower in fish exposed to formalin under a MM diet than those not formalin-exposed). Furthermore, after 18 days of formalin exposure, it was evident a maintenance of the macroalgae protection (AF), as depicted on the capacity to avoid oxidative damage (as LPO and PO increased in fish under a standard diet, which was not recorded in MM-supplemented fish).

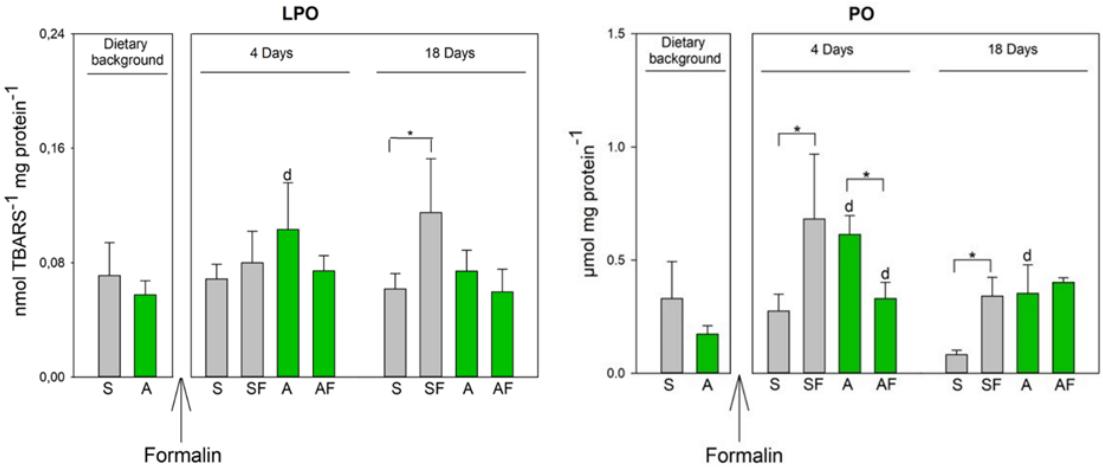

Figure 13: Levels of lipid peroxidation (LPO) and protein oxidation (PO), in the eyes of gilthead seabream (*Sparus aurata*) following 2 months of macroalgae dietary supplementation, as well as 4 and 18 days after a subsequent formalin treatment. Algae-supplemented groups (A) correspond to green bars, while groups fed with standard feeds (S) correspond to gray bars. Experimental groups submitted to formalin treatment are identified by abbreviations with an "F" after the letter identifying the dietary profile (i.e. SF and AF). The grey color represents the standard diet and the green color represents the macroalgae-enriched diet. Data were represented as mean ± standard error. Statistically significant differences between fish exposed to formalin and the respective unexposed group (for each feeding groups) are indicated by * ($p < 0.05$). Statistically significant differences between different dietary backgrounds (within formalin exposed fish and non-exposed fish) are identified by "d" ($p < 0.05$).

3.4. Acetylcholinesterase activity in the eyes

No significant alterations were found in the eyes' baseline AChE activity, meaning that after 2 months of the settlement of the different dietary backgrounds no differences were recorded between fish under a standard diet and a MM-enriched one. The lack of differences between dietary backgrounds were maintained along the experiment, regardless fish had been exposed to formalin or not.

Formalin was on the basis of an increase of AChE activity in the eyes of fish fed with a standard diet (SF), upon 4 days of exposure, which was not recorded in fish under a supplemented MM-diet (AF). Differently, after 18 days of exposure to formalin a decrease of AChE was found in fish under a standard diet (SF), which was apparently counteracted in fish under a MM-enriched diet (AF) (no changes were perceived).

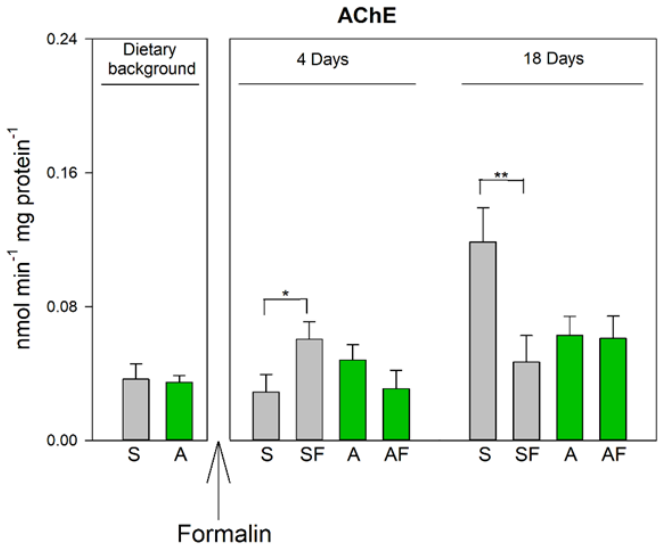

Figure 14: Activities of acetylcholinesterase (AChE) in the eyes of gilthead seabream (*Sparus aurata*) following 2 months of macroalgae dietary supplementation, as well as 4 and 18 days after a subsequent formalin treatment. Algae-supplemented groups (A) correspond to green bars, while groups fed with standard feeds (S) correspond to gray bars. Experimental groups submitted to formalin treatment are identified by abbreviations with an "F" after the letter identifying the dietary profile (i.e. SF and AF). The grey color represents the standard diet and the green color represents the macroalgae-enriched diet. Data were represented as mean ± standard error. Statistically significant differences between fish exposed to formalin and the respective unexposed group (for each feeding groups) are indicated by * ($p < 0.05$), ** ($p < 0.01$). Statistically significant differences between different dietary backgrounds (within formalin exposed fish and non-exposed fish) are identified by "d" ($p < 0.05$).

3.5. Enzymatic and non-enzymatic antioxidants in the brain

Regarding to the baseline levels in the brain, after the dietary background phase that lasted 2 months, CAT and SOD showed an activity decrease in fish fed with a MM-enriched diet (AF). No changes were recorded for the remaining analyzed antioxidants, namely on GPx, GST and GSHt after 2 months of the dietary background settlement. Later, upon 4 days of fish exposure to formalin it was notorious a significant increase of several antioxidants in the brain, such as CAT, GST and GSHt. These changes were concomitantly recorded in fish exposed to formalin and in non-exposed fish, both MM-supplemented (A and AF). Differently, no differences were found for SOD and GPx upon 4 days of formalin exposure. After 18 days of formalin exposure, levels of some antioxidants in the brain were more pronounced, with CAT, SOD and GPx showing significant higher activities in control fish fed with MM (A), meaning an improvement of the antioxidant protection, which was also perceived for CAT and

GPx in fish exposed to formalin (AF). In the same sampling period, levels of brain GSHt increased significantly in fish fed with MM, even without exposure to formalin.

After the formalin exposure, a delayed impact was perceived in the brain, since formalin effects were only detected 18 days after exposure. At this time, formalin induced an enhancement of SOD activity in the brain of non-supplemented individuals (SF) that was prevented by MM supplementation (AF). The glutathione cycle in brain was significantly altered upon formalin exposure (SF), since a notorious increase on GST activity and GSHt content was recorded. Interestingly, these changes occurred concomitantly in fish supplemented with MM (AF) and in individuals under a standard diet (SF), suggesting that MM were not able to prevent formalin-induced changes.

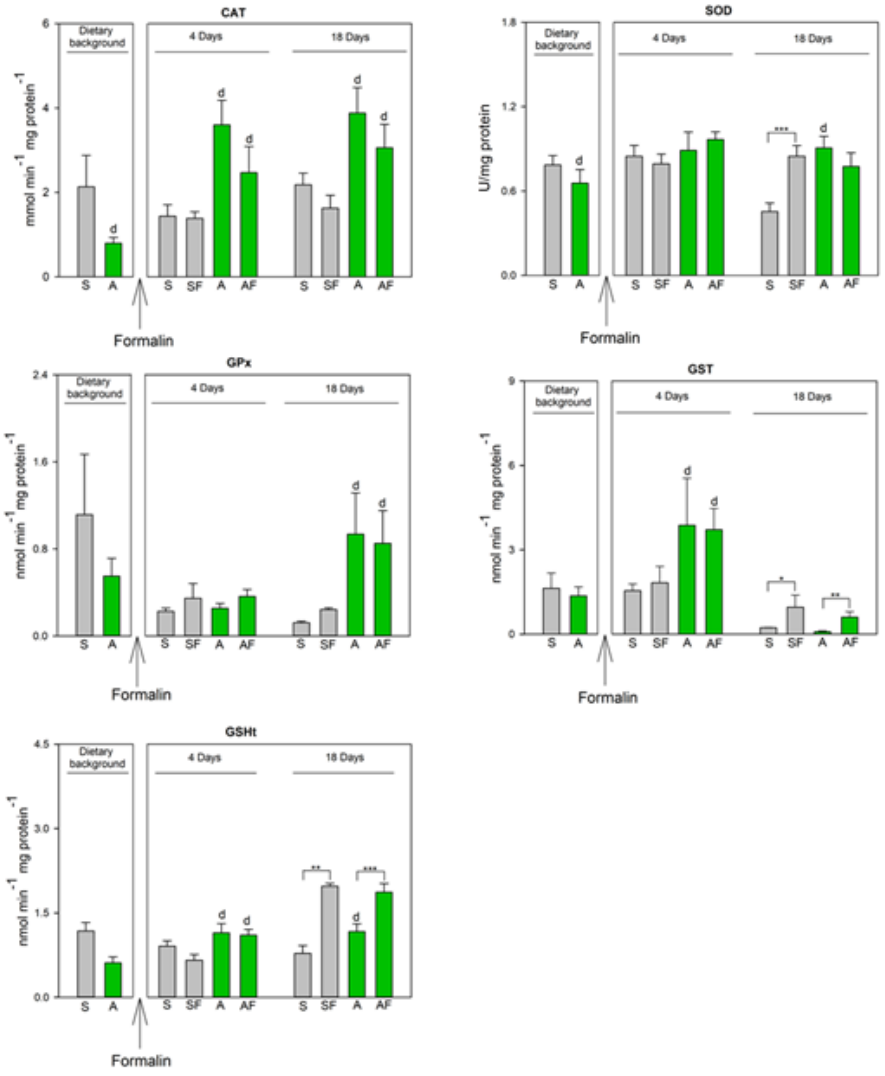

Figure 15: Activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione -s-transferase (GST), together with the levels of total glutathione (GSHt), in the brain of gilthead seabream (*Sparus aurata*) following 2 months of macroalgae dietary supplementation, as well as 4 and 18 days after a subsequent formalin treatment. Algae-supplemented groups (A) correspond to green bars, while groups fed with standard feeds (S) correspond to gray bars. Experimental groups submitted to formalin treatment are identified by abbreviations with an "F" after the letter identifying the dietary profile (i.e. SF and AF). The grey color represents the standard diet and the green color represents the macroalgae-enriched diet.Statistically significant differences between fish exposed to formalin and the respective unexposed group (for each feeding groups) are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p< 0.001$). Statistically significant differences between different dietary backgrounds (within formalin exposed fish and non-exposed fish) are identified by "d" ($p < 0.05$).

3.6. Damage of lipids in the brain

No significant differences were found for brain lipid peroxidation between fish supplemented with MM and non-supplemented individuals after 2 months of dietary supplementation (S and A). Four and eighteen days after the formalin exposure, it was found the same pattern than in the dietary background, meaning that no differences were recorded for LPO between supplemented and non-supplemented fish, regardless the formalin exposure. Nevertheless, after 18 days of formalin exposure, damage of the brain lipids in fish under a standard diet was noticed (SF). Interestingly, this pattern was not recorded in fish under a MM supplemented diet (AF) (a decrease was even perceived), suggesting protection afforded by MM.

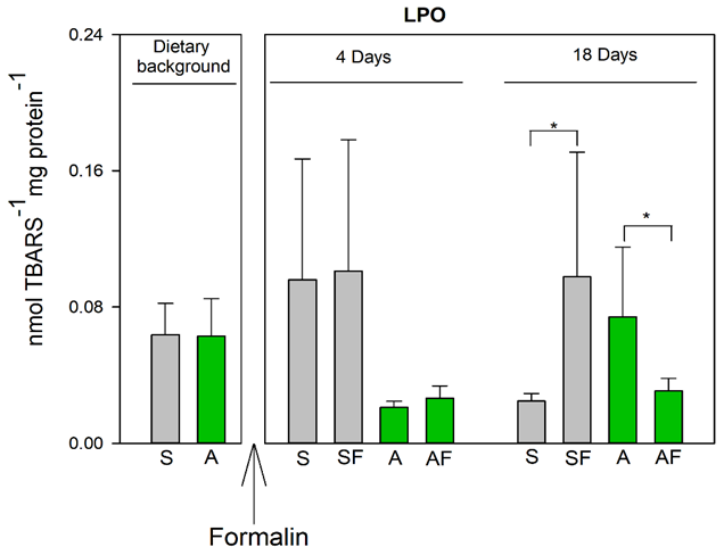

Figure 16: Activities of lipid peroxidation (LPO) in the brain of gilthead seabream (*Sparus aurata*) following 2 months of macroalgae dietary supplementation, as well as 4 and 18 days after a subsequent formalin treatment. Algae-supplemented groups (A) correspond to green bars, while groups fed with standard feeds (S) correspond to gray bars. Experimental groups submitted to formalin treatment are identified by abbreviations with an "F" after the letter identifying the dietary profile (i.e. SF and AF). The grey color represents the standard diet and the green color represents the macroalgae-enriched diet. Data were represented as mean ± standard error. Statistically significant differences between fish exposed to formalin and the respective unexposed group (for each feeding groups) are indicated by * ($p < 0.05$). Statistically significant differences between different dietary backgrounds (within formalin exposed fish and non-exposed fish) are identified by "d" ($p < 0.05$).

3.7. Acetylcholinesterase activity in the brain

No significant differences were recorded in brain AChE activity after the dietary background phase of 2 months between the S and A treatment. Four days upon formalin exposure, it was notorious

an increase on the brain AChE activity in fish that were fed with MM and exposed to formalin (AF), which had also occurred in control fish (A). This pattern was not found 18 days after formalin exposure, since an enhancement of AChE activity was found only in control fish supplemented with MM (A).

Formalin-induced alterations were only noticed 18 days after exposure, with this toxicant inducing an imbalance in the cholinergic homeostasis (as perceived by the increase activity on AChE in SF), which was not barred by MM enrichment feed (AF).

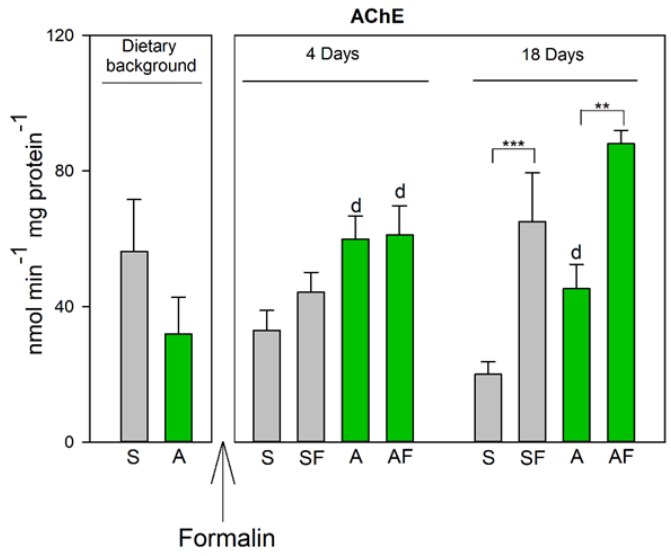

Figure 17: Activities of acetylcholinesterase (AChE) in the brain of gilthead seabream (*Sparus aurata*) following 2 months of macroalgae dietary supplementation, as well as 4 and 18 days after a subsequent formalin treatment. Algae-supplemented groups (A) correspond to green bars, while groups fed with standard feeds (S) correspond to gray bars. Experimental groups submitted to formalin treatment are identified by abbreviations with an "F" after the letter identifying the dietary profile (i.e. SF and AF). The grey color represents the standard diet and the green color represents the macroalgae-enriched diet. Data were represented as mean ± standard error. Statistically significant differences between fish exposed to formalin and the respective unexposed group (for each feeding groups) are indicated by * ($p < 0.05$), ** ($p < 0.01$). Statistically significant differences between different dietary backgrounds (within formalin exposed fish and non-exposed fish) are identified by "d" ($p < 0.05$).

4. DISCUSSION

This study addressed, for the first time, the benefits of using a marine macroalgae (MM)-supplemented diet to improve the antioxidant condition and neurotransmission of the *Sparus aurata* (gilthead seabream) eyes and brain. MM are widely known for their antioxidant abilities (Souza et al., 2012; Woo et al., 2013) and, thus they may protect fish eyes and brain from the deleterious effects of endogenous ROS, as well as those produced upon exposure to an external challenge (*e.g*. contaminant; UV-light). Additionally, benefits of MM on neurotransmission have been largely described, particularly in mammals (El Gamal, 2010; Harnedy and Fitzgerald, 2011; Pangestuti and Kim, 2013; Alghazwi et al., 2016). In this dissertation it was, specifically, tested the hypothesis that providing a MM-enriched diet to farmed gilthead seabream, over 2 months, it could enhance the antioxidant defenses and improve the neurotransmission under fish baseline conditions. An additional hypothesis was considered, which was testing the benefits of a MM-enriched diet to those organs upon an exposure of fish to formalin, a commonly used disinfectant in aquaculture. As an ultimate goal, this work aimed to point out to the regular use of a MM-enriched diet in the *S. aurata* farming, related with its potential health benefits.

The current diet consisted in a mix of three macroalgae species from different taxonomic groups, namely *Fucus vesiculosus*, *Gracilaria gracilis* and *Ulva rigida*, at a final proportion of 5%. Under these conditions, no significant changes were recorded on weight, total length and condition factor of the fish fed with a standard diet (group S) and a MM-enriched diet (group A) over the experimental time, suggesting that MM can be included in gilthead seabream diet without compromising growth rates, and therefore with no detrimental effects on fish production revenues. These results are in line with those of Peixoto et al. (2016) in the european bass (*Dicentrarchus labrax*), where no effects on fish growth were reported upon an enriched diet with a mixture of algae from the genera *Gracilaria, Fucus* and *Ulva,* or upon testing *Gracilaria* only. Other studies also highlighted the absence of negative effects of dietary MM in fish growth, pointing out for its incorporation on fish diet on a regular basis in aquaculture. These results are in line with those of Peixoto et al. (2016) in the european bass (*Dicentrarchus labrax*), since no effects on fish growth were reported upon an enriched diet with a mixture of algae from the genera *Gracilaria*, *Fucus* and *Ulva*, or testing *Gracilaria* only. Other studies also highlighted the absence of negative effects of a MM-enriched diet in fish growth, pointing out for its incorporation on fish diet on a regular basis in aquaculture. Examples of these studies were performed with 10 % of red macroalgae *Pterocladia* or 5 % of *Ulva* meal, where it was shown that these

macroalgae didn't affect negatively the growth and even improve the performance, feed utilization, nutrient retention and survival (Wassef et al., 2005). Batista (2018) demonstrated that the incorporation of MM in the diet, such as *Ulva rigida* and *Gracilaria* sp., in the diet of *Sparus aurata* did not affect the growth.

Accordingly, no differences on fish weight, length and condition index over the experimental time were found between fish under different diets (S, SF, A, AF), when exposed to formalin. This is in accordance with another study of Peixoto team (2016b) when a dietary MM supplementation was provided to a meagre species that was subjected to a bacterial infection. The MM supplementation used in the meagre can be useful to modulate the fish performances in order to match aquaculture rearing conditions. Furthermore, the same study, suggest that MM can be include in the meagre diet without compromising the growth rate.

In contrast, it was found a general evidence of altered antioxidant responses in gilthead seabream eyes and brain in fish fed with an MM-supplemented diet. Intriguingly, an enhancement of lipid and protein damage was recorded in the eyes of fish under a MM-enriched diet, raising some questions on the benefits of MM under baseline conditions. Moreover, an enhancement of AChE activity was found specifically for the brain in fish supplemented with MM, while no effect of diet was recorded in the eyes. In general, in the eyes the benefits of MM were more straightforward upon exposure of the fish to formalin, since it was evident the attenuation of formalin effects on antioxidants, damage indicators and AChE, both after 4 and 18 days upon exposure. Apparently, effects of formalin in the brain were delayed in relation to what was recorded for the eyes. In fact, no changes were recorded for brain antioxidants, damage indicators and AChE in the brain upon 4 days of formalin exposure, either in fish under a standard diet or MM-enriched feeds. Differently, formalin exposure was on the basis of antioxidants enhancements after 18 days of exposure, as well as AChE in the brain of fish fed with a standard diet, which, in general, was not reverted in fish under a MM-enriched diet. A single exception was found for LPO that was augmented in the brain of fish exposed to formalin when feeding a standard diet, while a decrease was found in the brain of fish under MM-enriched feeds.

In the following sections of the discussion, the neuroprotective effects of dietary MM in the eyes and brain of *S. aurata* will be discussed in detail under standard conditions and after formalin-induced toxicity. Evaluation of the neuroprotection afforded by MM comprised both the antioxidant condition of the tissues and neurotransmission status. Finally, a comparison of the vulnerability of fish eyes and brain to formalin-induced toxicity will be discussed, together with the differential protection

afforded by MM. This discussion will be made alongside with a list of potential biocompounds present in the three species of MM used in the fish diets, according to the literature.

4.1. Effects of dietary macroalgae on the antioxidant protection and neurotransmission condition of the eyes

After the dietary background phase that lasted 2 months, it was notorious an alteration of the baseline levels of the first line of antioxidants defense (i.e., CAT, SOD and GPx) in the eye tissues. Specifically, the eyes of MM-supplemented fish showed lower activities of CAT and SOD after 2 months of dietary supplementation, while GPx was enhanced. The lower levels of CAT and SOD are difficult to explain in the eyes of fish fed with MM. An enhancement of the endogenous levels of both enzymes would be expected, as MM are most probably a source of CAT and SOD cofactors, namely iron and manganese (Zhao et al., 2014; Wells et al., 2016), respectively. These metals are commonly found in MM (Wells et al., 2016) and could be absorbed by the fish intestinal epithelium (Bury, 2003), although the absorption mechanisms remain unclear so far. High levels of iron and manganese in the eyes could ultimately lead to an increase of endogenous CAT and SOD, but the opposite was found after 2 months of MM-supplementation. Differently, it was recorded and enhancement of the GPx activity in the eyes of fish supplemented with MM for 2 months. Glutathione peroxidase is a selenoenzyme that uses selenium as a cofactor (Flohe et al., 1973). It has been showed that MM species can be a valuable source of dietary selenium (Schiavon et al., 2017), which could be hypothetically on the basis of the GPx enhancement in the eyes. Current findings are in line with previous observations in rats, which were fed with an extract of *Ulva lactuca* enriched in sulfated polysaccharides. An increase of the GPx levels was recorded in the liver of rats under that diet (Hassan et al., 2011), this increase was represented by an improve of 77 % in the GPx when compared with the control group. In addition to the MM cofactors that fish can probably absorb, these MM species can be a source of other bioactive compounds that can indirectly interfere positively with the antioxidant system. For instance, fucosterol and sterols present in a brown MM *Padina gymnospora* demonstrated a capacity to induce an increase of GPx activity (Kristinsson, 2014). Moreover, vitamins (*e.g*. vitamin C), are essential for the good eyes functioning and these compounds are also found in MM (Michalak and Chojnacka, 2015). Despite that, dietary MM did not have effects on the activities of GST and GR, or in total GSH levels of the eyes after 2 months of dietary supplementation. Accordingly, no differences were found between fish supplemented with MM and those under a standard diet regarding damage of lipids and proteins, as

well as AChE activity. Overall, after the two months of the dietary background settlement, no major benefits of MM could be discerned in the eyes of fish under a supplemented diet. Interestingly, (Peixoto et al., 2016) had even found an increase in the GR activity in liver tissue of European seabass (*Discentrarchus labrax*) fed with a mix diet composed by *Gracilaria*, *Ulva* and *Fucus*. Furthermore, the same study reports an increase in the LPO levels in the individuals fed with the MM mix. It was also found non-significant differences in the liver GST activity in the liver of *D. labrax* fed with the MM mixture (Peixoto et al., 2016).

In control fish (non-exposed to formalin), different diets were on the basis of distinct patterns regarding the eyes antioxidant defenses and damage indicators over time, specifically after 4 and 18 days since the time-point correspondent to formalin exposure. At day 4, it was found a decrease of SOD, GPx and GST activities, while lipid peroxidation and protein oxidation were significantly augmented. In general, these data did not underpinned the benefits of MM supplementation on the antioxidant condition of the gilthead seabream eyes (oxidative stress was even recorded), which is in fact in accordance with results after the 2 months of dietary background settlement when a poor variation of the measured endpoints was noticed. Also, Magnoni et al. (2017) reported a decrease of the hepatic GPx activity in *Sparus aurata* fed with *Gracilaria* (one of the genus used in the current work). Moreover, Peixoto et al. (2016), had already reported an enhancement of lipid peroxidation in seabass fed with a MM supplemented diet, as described above. By the time, the authors suggested that seaweed supplementation might increase the lipid layer degradation. Additionally, Queiroz et al. (2014) also reported a significant increase of lipid peroxidation in the liver of gilthead seabream upon 4 supplemented diets (Phaeophyta, Chlorophyta, Rhodophyta and a mix of the three) and in the *Gracilaria* treatment when compared to control fish. This conclusion was in accordance with another study that revealed a dose-dependent inhibition in lipid accumulation in cells treated with *Gracilaria verrucosa* extracts (Woo et al., 2013). Furthermore, MM supplementation may be conferring a reinforcement antioxidant action with the aim of strengthening the organism and this leads first to an increase in the levels of oxidative damage, which then drops to baseline levels. The eyes of fish supplemented with MM and exposed to formalin exhibited the same variation pattern for SOD (*i.e.*, a significant decrease). Contrastingly, a reduction of the protein oxidation was found in the eyes of fish exposed to formalin under a supplemented diet with MM, fostering the hypothesis that some protection could be afforded to the eyes of fish under a formalin challenge.

At day 18 since the formalin challenge, a significant increase of antioxidant defenses was recorded in the eyes of fish supplemented with MM (control fish), being characterized by the enhancement of CAT, SOD, GPx and GST activities. This was noticed regardless an increase of protein oxidation. Also, Michalak and Chojnacka (2015) showed an increase in the CAT, SOD and GPx enzymes when rats were fed with a *U. lactuca* extract. Data on gilthead seabream eyes suggest that the enhancement of antioxidant protection afforded by MM could be time-dependent since a consistent increase of antioxidants was noticed after a more prolonged time of fish under an enriched-MM diet (2 months of dietary background settlement, plus 18 days). This enhancement of the antioxidant protection was found, even if it was not enough to counterbalance ROS production since protein oxidation had been recorded. Differently, almost no changes were found in the eyes of fish exposed to formalin and supplemented with MM (in comparison with exposed fish to formalin under a standard diet), suggesting that the protection afforded by dietary MM was displaced by formalin exposure.

Moreover, MM-enriched feed did not alter significantly the neurotransmission in the eyes of fish under baseline conditions (fish non-exposed to formalin) or upon exposure to an exogenous challenge. However, the literature showed that MM could have inhibitory effects in the cholinesterase's of rats (Pangestuti and Kim 2011). Nevertheless, one of the compounds tested by Yoon et al. (2008), phlorotannins presented in *Ishige okamurae* showed a capacity to induce the AChE activity in *in vitro* cells, given that it may have the capacity to mask the ChE and prevent the binding of the subtracts (Pangestuti and Kim, 2011).

Formalin had a notorious effect in the eyes of gilthead seabream under a standard diet, as perceived by changes on enzymatic and non-enzymatic antioxidants after 4 days of exposure. In fact, a significant decrease of GPx and GST was noticed, together with an enhancement of GR. Moreover, protein oxidation was increased. The later effect that is an indication of oxidative stress resultant from an excess of ROS, which were not properly counterbalanced by the antioxidant defenses, did not occurred in the eyes of fish under MM dietary supplementation. In fact, a decrease of protein oxidation was even recorded. In fish under a MM-enriched diet and exposed to formalin, an enhancement of total glutathione levels was found, suggesting that this antioxidant had probably a chief role on protein damage prevention. In fact, glutathione and the related enzymes are an important defense system of the eyes against oxidative stress (Ganea and Harding, 2006). Furthermore, the glutathione plays an important role in maintaining the normal hydration level and protects the integrity of the cellular membrane in the cornea (Ganea and Harding, 2006). Besides that, glutathione is distributed in the

retinal cells (Ganea and Harding, 2006). Other MM compounds (not addressed in this study) could had play a role in protecting the eye of fish exposed to formalin, particularly vitamins, such as the vitamin A, C and E.

Eighteen days after the formalin exposure, its toxicity could be still perceived in the eyes of the gilthead seabream under a standard diet, namely by a significant enhancement of lipid peroxidation and protein oxidation, pointing out to an excessive production of ROS, as already observed after 4 days of exposure. Formalin was probably on the basis of a depletion of total glutathione in the eye tissues, resulting in cellular damaging, which is in accordance with the lower levels of GSH that were found. The antioxidant GSH system is an important target in mediating the toxicity of several exogenous compounds. GSH is the most abundant intracellular low molecular weight thiol compound in all tissues, including the CNS (Dringen et al., 2000) Its reducing capacity is determined by the nucleophilic properties of its thiol group and its antioxidant role is sustained by the presence of several enzymes that catalyze its interaction with endogenous and xenobiotic electrophilic molecules (Farina et al., 2011). Of particular importance, glutathione peroxidase (GPx) and glutathione reductase (GR) are central enzymes involved with detoxification of peroxides and reduction of glutathione disulphide (oxidized glutathione; GSSG), respectively (Dringen et al., 2000). Activities of these enzymes, as well as the maintenance of a normal thiol status, represented mainly by the GSH/GSSG ratio, are essential for protecting cells against oxidative damage. Other toxicants, such as mercury, are known to form a complex that is easily excretable from the cells, leading to a decrease of GSH levels and ultimately to oxidative stress (Franco et al., 2007). The formalin toxicity mechanism was not described so far, but a similar interaction with GSH could be speculated at the light of current findings. Formalin when reaches the cells reacts with the proteins and with the lipids (Leal et al., 2016). This reaction will probably lead to the formation of lipid and protein damage. One study also found an increase of LPO and PO in rainbow trout (*Oncorhynchus mykiss*) tissues, namely the heart, concluding that cytotoxic effects are close related with the formaldehyde dose, damage increases with increasing formaldehyde dose (Tkachenko et al., 2015). The eyes of fish supplemented with MM did not exhibit cellular damage, since no lipid peroxidation or protein oxidation was found, pointing out to the benefits of MM on oxidative stress prevention when compared with the non-supplemented fish. An association with the enhancement of GR activity could be speculated. A previous study with *U. lactuca* diet has shown that macroalgae-enriched fed limited the lipid peroxidation process (Michalak and Chojnacka, 2015), which is in accordance with current findings.

Formalin induces, at four days after the exposure, the cholinergic activity in the eyes of the fish feed with standard diet. Zendehdel et al. (2016) showed that the increase in AChE is correlated with the formaldehyde exposure. However, a different tendency was found at the day 18, where formalin impairs the AChE activity. These results were recorded in a study with *Eisenia andrei* that also showed an inhibition when exposed to formalin (Velki et al., 2013). Therefore, in both periods it was evident a persistence of macroalgae protection in neurotoxicity symptoms here analyzed by AChE activity. Marine macroalgae have compounds with the capacity of modulate the cholinergic activity (Suganthy et al., 2010). For example, the AChE inhibition promoted by the biological compounds present in the MM plays an important role enhancing the cholinergic neurotransmission (Hodges, 2006).

4.2. Effects of dietary macroalgae on the antioxidant protection and neurotransmission condition of the brain

After two months of the dietary background settlement, the activities of CAT and SOD were lower in the brain of fish feed with MM, as previously reported for the eyes. Also, accordingly with the above section, these reductions did not uncover *per se* the benefits of dietary MM to the fish brain. Furthermore, the brain tissue are protected by a barrier (brain-blood barrier, BBB) that can regulate the entrance of cofactors, that are essential to these enzymatic antioxidants (Wells et al., 2016). For example, the BBB can limit the entrance of iron (essential for the catalase), in the way to avoid the iron overload (Mills et al., 2010). Later, 4 and 18 days since the formalin exposure, similar variation patterns were, in general, found for antioxidants, lipid peroxidation and AChE activity between the brain of fish supplemented with MM regardless the formalin exposure (meaning that treatments A and AF showed identical differences from S and SF, respectively). In fact, an increase of CAT activity was commonly found in the brain of fish under dietary MM either in exposed (SF and AF) or control fish (S and A) after 4 and 18 days of exposure. Another example, is the increase of GST activity and total glutathione at day 4 in the brain of fish supplemented with MM, regardless exposed or not to formalin (treatments A and AF). Even because, it is known that the brain has a high activity of glutathione enzymes (Forshammar et al. 2011) and the MM are an important source of glutathione (GSH) resulting in an significative increase in the glutathione enzymes present in the fish (Michalak and Chojnacka, 2015), leading to an increase of this antioxidant in the brain of fish fed with MM supplementation. Furthermore, the possibly absence of significant differences in brain GPx activity after 4 days of formalin exposure between supplemented and non-supplemented can be, probably explained, due to the significantly

increase in the CAT activity in the supplemented fish, since, according to Ryan et al. (2008), these two enzymes have some overlap function and when the organism produces a sufficient amount of CAT or GPx there is no need for producing of GPx or CAT, respectively. The same common pattern was recorded for AChE at day 4, or for GPx at day 18. Glutathione peroxidase activity is influenced by the feeding behaviour and carnivorous fish has tendency to have a low activity of this enzyme when compared with fish that had an herbivorous content in their feed (Martínez-Álvarez et al., 2005). As already mentioned, this enzyme is a selenoenzyme and this means that need selenium as a cofactor (Flohe et al., 1973) and marine macroalgae are an important source of selenium (Schiavon et al., 2017), but the blood-brain barrier may be hindering the selenium entrance in the brain. However, over the time the selenium uptake may be more significative, reflecting an increase in the GPx activity at this time. Identical variations seem to indicate that dietary MM was on the basis of an increase of the antioxidant protection in the gilthead seabream brain in an extent that it was not even compromised/decreased by the formalin exposure. This is a very interesting result, underpinning the benefits of MM to the fish brain functioning. Nevertheless, a few exceptions were found, particularly after 18 days since formalin exposure. The increase of SOD and total GSH noticed in the brain of control fish supplemented with MM was not recorded in supplemented fish when exposed to formalin. Accordingly, the increase of AChE activity in supplemented control fish was not found in formalin-exposed ones. This is an intriguing result since negative effects in the brain would be expected by the elevated levels of AChE activities, as documented in mice. Multi-site dysfunctions related with elevated AChE activities were reported in mice, ranging from cognitive deficiencies, intensified neuropathology markers for neurodeterioration in the brain and neuromotor impairments. Natarajan et al. (2009) used extracts of several MM (*Ulva reticulata, Turbinaria conoides, Turbinaria ornata, Hypnea valentiae*, among others) in order to show the inhibition of AChE extracted from the brain of Nile tilapia. Furthermore, another study with Nile tilapia showed the inhibitory capacity of methanol extract of *Hypnea valentiae* and *Ulva reticulata* in the muscle AChE (Suganthy et al., 2010).

Contrarily what was observed in the eyes, formalin induced a late effect in the brain. Toxicity of formalin in the brain of fish under a standard diet was found, mainly related with the enhancement of lipid peroxidation. This result can be partly explained by the existence of a high concentration of lipids in the brain (Adibhatla and Hatcher, 2007). Apparently, the increase of the antioxidant defenses SOD, GST and GSH was not able to prevent brain lipid peroxidation in fish fed with a standard diet. Contrastingly, the brain of fish under a MM-enriched diet did not have an enhancement of lipid

peroxidation (but even a decrease), indicating that MM afforded an increase of antioxidant defenses that were able to prevent oxidative stress. Since common variation patterns were found for all the measured antioxidants in the brain of fish under a MM-enriched diet and those fed with standard aquafeeds (except SOD that was higher in the brain of fish from SF treatment in comparison with S), it is difficult to discern which compounds could had been on the basis of the highest protection in fish feed with MM. The species used to enrich the aquafeeds (namely *Fucus vesiculosus*, *Ulva rigida* and *Gracilaria gracilis*) have important ingredients that may contribute to improve brain functioning and eventually preventing lipid peroxidation, namely the PUFA that are essential to the brain function (Nowak, 2013) and have a high antioxidant capacity. Other compounds, like phlorotannins or sterols, have the capacity to remove the ROS preventing the damage in the tissue (Barbosa et al., 2014). The phycocyanin existent in the MM had a capacity to confer a neuroprotection to the fish brain, for example, in vitro studies showed a capacity of this compound to scavenge various oxygen radical and consequently inhibit lipid damage (Barbosa et al., 2014). Furthermore, the sulfated galactans present un the *Porphyra* spp. can reduce significantly the levels of malondialdehyde (MDA) that are a marker of lipid peroxidation (Mohamed et al., 2012). It was reported the protection of polysaccharide in mice against the lipid peroxidation (Zhang et al., 2017). Besides that, the MM sulfated polysaccharides have proprieties that can neutralize reactive species and prevent the oxidative damage (Kristinsson, 2014).

Formalin exposure was on the basis of an enhancement of AChE activity after 18 days of exposure. This increase was found in the brain of fish feed with a standard diet, and was not altered in the brain of fish supplemented with MM. The negative effects of an increase of AChE activity in the brain were already described at the light of mice findings, and MM did not seem to be able to attenuate these effects related with formalin exposure.

4.3. A comparative analysis on the vulnerability of eyes and brain to formalin and the potential benefits of macroalgae

Fish are exposed to formalin by immersion in water spiked with this compound at a concentration range of 100 - 250 mg L^{-1} however, the concentration applied to the treatment varies between the fish age, water temperature or water flux (Leal et al., 2016). There are some divergent findings on the safety of the concentrations of formalin used during this common aquaculture procedure. Some studies revealed toxic effects in the gills related with their direct contact with formalin

(Shepherd and Bromage, 2001). Reported effects were observed on salmon gills exposed to formalin. Similarly, the fish eyes are in direct contact with waterborne formalin, and therefore toxicity is highly expected. In fact, in fish under a standard diet, formalin had led to an increase of protein oxidation, both after 4 and 18 days of exposure, as well as lipid damage after 18 days of exposure (Table 3). This was followed by changes on several antioxidants levels, as described in section 4.1. Moreover, formalin altered the neurotransmission condition in the eyes after 4 and 18 days of exposure, as perceived by an enhancement of AChE activity and then by a decrease, respectively. Acetylcholinesterase is a paradigmatic endpoint of neurotransmission since the acetylcholine (ACh)-AChE system forms an important component of the nervous activity. Either the enhancement of AChE activity or its decrease has been associated with negative effects on the neurotransmission. In mice, elevated AChE activities had induced multi-site dysfunctions, as previously described. On the contrary, the inhibition of AChE in fish eyes would lead to the accumulation of the neurotransmitter acetylcholine (ACh) and, thus, to the eventual compromise of synaptic transmission. An interesting result of this dissertation, was that protein oxidation in the eyes, as well as alterations on AChE that were recorded in fish exposed to formalin under a standard diet, were not found in fish fed with MM. This finding suggests that MM compounds with antioxidant protection were absorbed at the intestinal epithelium reaching the eyes, as well as other molecules that could maintain AChE under optimal physiological levels. As detail in section 4.1, glutathione was probably one of these molecules that ultimately could act on ROS scavenging, and thus preventing protein damage. Regarding AChE,, Myung et al. (2005) showed that dieckol and phlorofucofuroeckol present in the brown macroalgae *Ecklonia cava* are directly related with the increment of the ACh through inhibition of AChE.

The effects of formalin in the brain were never investigated in fish. Considered that the exposure route to formalin is the water, a pertinent question is whether waterborne formalin can pose a big threat to the central nervous system of fish by entering the blood and ultimately reaching the brain after crossing the blood–brain barrier. This hypothesis was never addressed in fish, but can be discussed at the light of findings in humans for formaldehyde (the major component of formalin). Exposure to high concentrations of exogenous formaldehyde that exceeds the peripheral formaldehyde oxidation capacity will elevate the normal tolerable concentration of formaldehyde in the blood and could lead to neural damage (Tulpule and Dringen, 2013). Indeed, exposure to exogenous formaldehyde has been reported to cause neurotoxicity in humans and animals and the extent of damage depends on the dose of formaldehyde and the duration of the exposure (Tulpule and Dringen,

2013). At this light, an increase of ROS could be expected at the brain of the gilthead seabream, leading eventually to lipid damage, if the antioxidant protection is not able to counterbalance ROS production. This hypothesis was confirmed after 18 days of formalin exposure in the brain of fish under a standard diet, where lipid peroxidation was recorded, despite the increase of SOD, GST and GSHt. Interestingly, oxidative stress as perceived by the enhancement of lipid peroxidation was not recorded in the brain of fish supplemented with MM. As hypothesized for the eyes, the benefits of a MM-enriched diet described here for the brain, would imply a significant absorption of compounds with antioxidant properties at the intestinal level. It is important to highlight that effects of formalin in the brain were delayed in relation to what was observed for the eyes. While significant effects were found in the brain only after 18 days after formalin exposure, the oxidative stress was recorded in the eyes just after 4 days of exposure.

A MM-enriched diet of fish was able to counteract with oxidative stress related effects both in the eyes and brain (i.e., increase of protein oxidation in the eyes after 4 and 18 days of exposure and lipid peroxidation after 18 days, as well as an enhancement of brain lipid peroxidation at day 18) (Table 3). Contrastingly, different patterns were recorded for the activity of AChE, since while in the eyes the effects of formalin were reverted in fish fed with MM, that was not recorded in the brain (an increase activity of AChE was simultaneously recorded in the brain of supplemented fish with MM and those under a standard diet).

Table 3: Synopsis of the eyes brain oxidative stress profiles upon fish exposure to formalin. Significant alterations are marked by up and down arrows meaning increased or decreased levels of the parameter, respectively. Grey arrows represent the standard diet and the green arrows represent the MM-enriched diet.

	Eyes				Brain			
Time (days)	4 Days		18 Days		4 Days		18 Days	
condition	SF	AF	SF	AF	SF	AF	SF	AF
CAT			↑	↓				
SOD				↓			↑	
GPx	↓	↓	↑					
GST	↓	↓	↑				↑	↑
GSHt		↑	↑				↑	↑
GR	↑			↑				
LPO			↑				↑	↓
PO	↑	↓	↑					
AChE	↑	↓					↑	↑

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The results of this dissertation may have implications to the aquaculture sector, specifically for the gilthead seabram (*Sparus aurata*) farming since dietary marine macroalgae (MM) supplementation may be used to improve the antioxidant protection of the eyes and brain, as well as neurotransmission, particularly under exogenous challenging conditions (in this case formalin exposure, a commonly used disinfectant). Dietary MM supplementation with 5% of a mixture of *Ulva rigida*, *Gracilaria gracilis* and *Fucus vesiculosus* had no negative impact on the growth performance of the fish after 2 months that corresponded to the settlement of dietary background (namely with a standard diet and with a MM-enriched diet), as well as after a longer period that lasted 18 days when the evaluation of the effects of exposure to formalin was investigated. Interestingly, the benefits on the antioxidant protection afforded to the fish eyes were not completely evident under baseline conditions (i.e. without an external challenge). By the contrary, oxidative stress had probably occurred, as underpinned by the enhancement of lipid and protein damage in the eyes of fish of treatment A (control fish) after 4 days of formalin exposure. Moreover, no evidences on the benefits to the eyes neurotransmission, as addressed by AChE had been recorded related with a MM-enriched diet alone, as well. Benefits of MM dietary supplementation on the eyes of control fish (unexposed to formalin) were probably time-dependent, since an increase of the antioxidant protection was found later in the experiment (after 2 months of the dietary settlement plus 18 after formalin exposure), while no indication of damage was recorded. An exacerbation of the benefits of dietary MM supplementation was found after formalin exposure, since the effects of this toxicant in the eyes were significantly attenuated in fish under that diet. This was possible related with a higher availability of GSH, to counterbalance ROS produced by formalin. Besides that, the absence of changes on AChE in the eyes of fish fed with MM and exposed to formalin, in opposition to those under a standard diet, suggests benefits of MM diet on neurotransmission. In the brain, dietary MM seems to afford antioxidant protection under baseline conditions, which were not compromised by formalin exposure. Differently, no clear benefits could be discerned on brain neurotransmission related with dietary MM supplementation. Effects of formalin in the brain were delayed in relation to what was observed for the eyes, probably related with the BBB protection. Despite that, 18 days after formalin exposure the effects found in the brain of fish under a standard diet (particularly the enhancement of lipid damage) were prevented in fish supplemented with MM. Contrarily, no benefits were found for neurotransmission according to data on the proxy

AChE. At this light, the potential shielding properties of MM compounds against oxidative stress and neurotransmission disorders in the fish eyes and brain deserve further research under the aquaculture context, ultimately to mitigate the impact of the use of formalin and other routinely applied chemotherapeutic agents.

The current investigation can be very much enriched by other complementary approaches to clarify the benefits of dietary MM on fish neuronal and sensory condition in aquaculture. The use of a metabolomic approach can be useful to clarify the metabolites that are absorbed by the fish and consequently by the eyes and brain. Another approach that can be exploited, is the use of other marine macroalgae species or another diet combination, to elucidate the results obtain for the oxidative damage in the eyes and to find other MM with bioactive compounds that can be beneficial for the neuronal and sensory condition against the formalin exposure.

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7. Appendix

Table 4: Mann-Whitney U test for the weight, length and condition factor between the different treatments in dietary background, 4 days and 18 days. The grey color represents the standard diet and the green color represents the macroalgae-enriched diet.

		P-value		
	Treatment	Weight	Length	Condition factor (K)
Dietary background	S vs. A	> 0.05	> 0.05	> 0.05
4 Days	S vs. A	> 0.05	> 0.05	> 0.05
	S vs. SF	> 0.05	> 0.05	> 0.05
	A vs. AF	> 0.05	> 0.05	> 0.05
	SF vs. AF	> 0.05	> 0.05	> 0.05
	A vs. SF	> 0.05	> 0.05	> 0.05
	S vs. AF	> 0.05	> 0.05	> 0.05
18 Days	S vs. A	> 0.05	> 0.05	> 0.05
	S vs. SF	> 0.05	> 0.05	> 0.05
	A vs. AF	> 0.05	> 0.05	> 0.05
	SF vs. AF	> 0.05	> 0.05	> 0.05
	A vs. SF	> 0.05	> 0.05	> 0.05
	S vs. AF	> 0.05	> 0.05	> 0.05

Table 5: Mann-Whitney U test for the dietary background and Kruskal-Wallis one way analyses of variance for the (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR), total glutathione (GSHt), lipid peroxidation (LPO), protein oxidation (PO) and acetylcholinesterase (AChE) at the end of the 4 and 18 days after the formalin bath between the different treatments in the eyes. Significative results are marked in bolt. The grey color represents the standard diet and the green color represents the macroalgae-enriched diet.

		P-value								
	Treatment	CAT	SOD	GPx	GST	GSHt	GR	LPO	PO	AChE
Dietary background	S vs. A	**< 0.05**	**< 0.05**	**< 0.05**	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
4 Days	S vs. A	> 0.05	**< 0.05**	**< 0.05**	**< 0.05**	> 0.05	> 0.05	**< 0.05**	**< 0.05**	> 0.05
	SF vs. AF	> 0.05	**< 0.05**	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	**< 0.05**	> 0.05
	S vs. SF	> 0.05	> 0.05	**< 0.05**	**< 0.05**	> 0.05	**< 0.05**	> 0.05	**< 0.05**	**< 0.05**
	A vs. AF	> 0.05	> 0.05	**< 0.05**	**< 0.001**	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
18 Days	S vs. A	**< 0.05**	**< 0.05**	**< 0.05**	**< 0.05**	> 0.05	> 0.05	> 0.05	**< 0.05**	> 0.05
	SF vs. AF	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	**< 0.05**	> 0.05	> 0.05	> 0.05
	S vs. SF	**< 0.05**	> 0.05	> 0.05	**< 0.05**	**< 0.05**	> 0.05	**< 0.05**	**< 0.05**	**< 0.01**
	A vs. AF	**< 0.05**	**< 0.05**	> 0.05	> 0.05	> 0.05	**< 0.05**	> 0.05	> 0.05	> 0.05

Table 6: Mann-Whitney U test for the dietary background and Kruskal-Wallis one way analyses of variance for the (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), total glutathione (GSHt), lipid peroxidation (LPO) and acetylcholinesterase (AChE) at the end of the 4 and 18 days after the formalin bath between the different treatments in the eyes. Significative results are marked in bolt. The grey color represents the standard diet and the green color represents the macroalgae-enriched diet.

		P-value						
	Treatment	CAT	SOD	GPx	GST	GSHt	LPO	AChE
Dietary background	S vs. A	**< 0.05**	**< 0.05**	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
4 Days	S vs. A	**< 0.05**	> 0.05	> 0.05	**< 0.05**	**< 0.05**	> 0.05	**< 0.05**
	SF vs. AF	**< 0.05**	> 0.05	> 0.05	**< 0.05**	**< 0.05**	> 0.05	**< 0.05**
	S vs. SF	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
	A vs. AF	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
18 Days	S vs. A	**< 0.05**	**< 0.05**	**< 0.05**	> 0.05	**< 0.05**	> 0.05	**< 0.05**
	SF vs. AF	**< 0.05**	> 0.05	**< 0.05**	> 0.05	> 0.05	> 0.05	> 0.05
	S vs. SF	> 0.05	**< 0.001**	> 0.05	> 0.05	**< 0.01**	**< 0.05**	**< 0.001**
	A vs. AF	> 0.05	> 0.05	> 0.05	**< 0.01**	**< 0.001**	**< 0.05**	**< 0.01**

Table 7: Bioactive compounds with antioxidant capacity present in the three marine macroalgae classes.

	Compound	Species	Model	Reference
Antioxidant capacity	Antheraxanthin	Rodophyta	Rat	Schubert et al. 2006
	β - carotene	Rodophyta	Mice	Lohrmann et al. 2004
	Bromophenols	Rodophyta	Macroalgae extract	Xu et al. 2009
	Carotenoids	Phaeophyta	Macroalgae extract	Miyashita et al. 2011
	Catechin	Chlorophyta	Macroalgae extract	Devi et al. 2008
	Flavonoids	Rodophyta	Macroalgae extract	Yuan et al. 2005
	Fucoidan	Phaeophyta	Macroalgae extract	Chattopadhyay et al. 2010
	Fucoxanthin	Phaeophyta	Sea squirt	Sachindra et al. 2007
	Galactans	Chlorophyta Phaeophyta Rodophyta	Macroalgae extract	Costa et al. 2010
	Melatonin	Chlorophyta	Macroalgae extract	Fang et al. 2002
	Phlorotannins	Phaeophyta	Macroalgae extract	Ye et al. 2008
	Phycoerythrin	Rodophyta	Rat	Romay et al. 2003
	Phycocyannin	Rodophyta	Rat	Yabuta et al. 2010
	Polyunsaturated fatty acid	Chlorophyta Phaeophyta Rodophyta	Human	Ruxton et al. 2004

Table IV (continuation)

	Compound	Species	Model	Reference
Antioxidant capacity	Stypodiol	Phaeophyta	Macroalgae extract	Nahas et al. 2007
	Sulphated galactans	Phaeophyta Rodophyta	Macroalgae extract	Rocha De Souza et al. 2007
	Sulphated	Phaeophyta	Rat	Josephine et al. 2008
	Sulphated polysaccharide	Phaeophyta	Macroalgae extract	Vijayabaskar et al. 2012
	Taurine	Rodophyta	Human	Lourenço and Camilo 2002
	Terpenoids	Phaeophyta	Macroalgae extract	Foti et al. 1994
	Vitamin A	Chlorophyta Phaeophyta Rodophyta	Macroalgae extract	Kumar et al. 2008
	Vitamin E	Chlorophyta Phaeophyta Rodophyta	Macroalgae extract	Sealey and Gatlin 2002
	Zeaxantin	Phaeophyta	Macroalgae extract	Schubert et al. 2006